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Development and Characterization of Edible Coatings to the Preservation of Cheese Quality

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Título da tese

Desenvolvimento e Caracterização de Novos Revestimentos Edíveis para a Preservação da Qualidade de Queijos

Orientador

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ABSTRACT

Conventional synthetic packaging films have led to serious ecological problems due to their non-biodegradability. New natural polymeric materials have been studied in order to replace the conventional packaging materials. Being so, the future generation of packaging materials will be derived from renewable and biodegradable sources.

Edible coatings and films, in particular, can provide additional protection for food, being a fully biodegradable and environmental friendly packaging system; and can be combined with antimicrobial, antifungal and antioxidant substances found in many natural sources. They have been extensively applied in fruits and vegetables, however hardly explored in dairy products.

Based in these ideas, the main purpose of this thesis was the development and characterization of new edible coatings for cheese preservation. The research was based in a sequence of tasks, which started by the study of a method for the extraction of galactomannans from non-traditional sources (*Gleditsia triacanthos*, *Caesalpinia pulcherrima* and *Adenanthera pavonina*) and determination of their physicochemical properties. Phenolic and antioxidant compounds were recovered during extractions and their utilization as possible functional compounds to be introduced in edible films was evaluated. The ability of those galactomannans to act as main material for edible coatings and films was studied simultaneously with a commercial polysaccharide (chitosan). Finally, different formulations of polysaccharide, plasticizer and corn oil were tested as coatings for cheese based on their wettability and physical properties. The influence of the coatings and storage temperature on gas transfer rates, chemical and microbiological properties of cheese was evaluated.

The work performed has shown that the extraction and purification methodology used could be applied successfully to three galactomannans giving a clear indication that it may be used for other galactomannan sources as well. The results have also shown that the extracted galactomannans present adequate physicochemical characteristics to be used in the food industry. During galactomannans extraction procedures it was possible to obtain phenolic and antioxidant compounds from ethanolic and aqueous extracts. These extracts have shown ability to be incorporated in galactomannan films, imparting them functional properties.

The incorporation of glycerol and corn oil in galactomannan and chitosan films has shown that the water affinity of the components of the coatings/films is one of the most influencing factors over their physicochemical properties. Furthermore, chitosan and galactomannan coatings/films showed to have different properties associated with their different structures, affecting principally the glass transition temperature, water vapour permeability and elongation-at-break values of the edible films.

The methodology used for coating selection has shown to be useful to obtain the best formulation to be applied on cheese. The application of chitosan and galactomannan coatings and storage temperature influences the cheese gas exchange rate, being galactomannan coatings the ones allowing a lower gas transfer rate. The use of a galactomannan coating together with a storage temperature of 4 °C showed to improve shelf-life parameters of cheese. The evaluation of the application method of the coating, despite presenting similar performances, when referring to weight and moisture loss and colour changes of the cheese, presents different efficiencies, being the brushing application the one where less coating forming solution is wasted.

In conclusion, galactomannans can be used as edible coatings for application on cheese and their properties can be tailored (within limits) by the addition of plasticizer and oil, turning these coatings into promising replacements for synthetic packaging materials.

RESUMO

Os revestimentos e embalagens sintéticas têm gerado problemas ecológicos devido ao facto de não serem biodegradáveis. Novos polímeros de origem natural têm sido estudados com o objectivo de substituir os materiais sintéticos e, espera-se que a nova geração de materiais para embalagens provenha de fontes renováveis e biodegradáveis.

Neste sentido, nos últimos anos os revestimentos e filmes edíveis surgiram como possíveis substitutos das embalagens sintéticas, através da utilização de fontes renováveis e biodegradáveis. Os revestimentos e filmes edíveis podem ser usados para aumentar o tempo de prateleira dos alimentos, onde pode também haver a adição de agentes antimicrobianos, antifugicos e antioxidantes possibilitando uma protecção mais eficaz. No entanto, e apesar de serem muito utilizados em frutos e vegetais, têm sido pouco estudados em produtos lácteos.

Neste seguimento, o principal objectivo da presente tese foi o desenvolvimento e caracterização de novos revestimentos edíveis para a conservação do queijo. Para a sua concretização começou por estudar-se um método de extracção de novas fontes de galactomananos (*Gleditsia triacanthos*, *Caesalpinia pulcherrima* e *Adenhantera pavonina*) e a sua caracterização. No decorrer das extracções, os compostos antioxidantes e fenólicos foram recuperados e testados como possíveis compostos funcionais para adicionar a filmes edíveis. Posteriormente, a possibilidade de utilizar esses galactomananos como revestimentos e filmes edíveis foi testada juntamente com um polissacarídeo commercial (quitosano). Diferentes formulações, com a variação das concentrações de polissacarídeo, plasticizante e óleo foram testados como revestimentos e filmes. Em seguida, foi avaliada a influência dos revestimentos edíveis na transferência de gases, e nas propriedades químicas e microbiológicas do queijo durante o armazenamento.

O trabalho realizado demonstrou que a metodologia de extracção e purificação utilizada pode ser aplicada com sucesso em três tipos de galactomananos, permitindo antever a sua aplicação a outras fontes de galactomananos. Os galactomananos obtidos apresentaram características físico-químicas adequadas para serem aplicados na indústria alimentar. Durante as extracções foi possível obter compostos fenólicos e antioxidantes dos extractos aquosos e etanólicos, que

mostraram poder ser incorporados em filmes edíveis de galactomananos, conferindo-lhes propriedades funcionais.

A adição de glicerol e óleo de milho aos revestimentos e filmes edíveis de galactomanano e quitosano mostrou que afinidade à água dos revestimentos e filmes edíveis é um dos factores que mais influencia as suas propriedades físico-químicas. Além disso, o revestimentos e filmes de quitosano e galactomanano mostraram diferentes propriedades associadas às suas distintas estruturas, afectando a temperatura de transição vitrea, a permeabilidade de vapor de àgua e os valores elongação à quebra dos filmes.

A metodologia utilizada na selecção do revestimento a ser aplicado no queijo mostrou ser útil para a obtenção da melhor formulação. A aplicação dos revestimentos de galactomanano e quitosano, assim como a temperatura, mostraram influenciar a transferência de gases do queijo, sendo o revestimento de galactomanano o que garantiu uma menor transferência de gases. O revestimento de galactomanano juntamente com uma temperatura de armazenamento de 4 °C mostrou ser a melhor opção para melhorar os parâmetros de tempo de prateleira do queijo. A avaliação do método de aplicação do revestimento no queijo mostrou, que apesar dos queijos apresentarem resultados de perda de água e diferenças de cor semelhantes, apresentam diferentes eficiências de aplicação, verificando-se que o método por pincelamento é o método onde menos revestimento é desperdiçado.

Em suma, os galactomananos podem ser utilizados como revestimentos para aplicação em queijo, as suas propriedades podem ser modificadas (dentro dos limites) recorrendo à adição de plasticizante e óleo de milho, apresentando-se como um substituinte promissor dos materiais sintéticos usados em embalagens.

LIST OF PUBLICATIONS

This thesis is based on the work presented in the following publications:

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Cerqueira, M.A.; Souza, B.W.S.; Simões J.; Teixeira, J.A.; Domingues, M.R.M.; Coimbra, M.A.; & Vicente, A.A. Structural and thermal characterization of galactomannans from non-conventional sources. *Carbohydrate Polymers* (*in Press*).

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Miguel A. Cerqueira, Bartolomeu W.S. Souza, Joana T. Martins, António A. Vicente. Improved hydrocolloid-based edible coatings/films systems for food applications. Carbohydrate Polymers: Development, Properties and Applications. Ashutosh Tiwari (Ed), Nova Science Publishers, Inc., New York, USA. (in Press).

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

LIST OF GENERAL NOMENCLATURE

Symbol

k_H – Huggins' coefficient

k_K – Kramers' coefficient

M/G – mannose to galactose ratio

C – solution concentration.

M_v – viscosity average molecular mass

m_i – initial mass of seeds

m_r – mass of recovered dry endosperm

m_f – mass obtained from the filtration and centrifugation

IC_{50} – concentration of the compounds that caused a 50 % inhibition of the radical scavenging activity.

R^2 – coefficient of determination

$\hat{y}_e$ – average of all experimental data points

A_f – accuracy factor

E – mean relative percentage deviation modulus

Y1– yield of extraction of polysaccharide pre-treatment

Y2 – yield of extraction of extraction and purification

Y3 – yield of extraction of for the global process

WVP – water vapour permeability

O_2P – oxygen permeability

CO_2P – carbon dioxide permeability

TS – tensile strength

EB – elongation-at-break

W_s – spreading coefficient

W_a – work of adhesion

W_c – work of cohesion

W_{sI} – spreading coefficient for experimental setup I

W_{sII} – spreading coefficient for experimental setup II

WVP_I – water vapour permeability for experimental setup I

WVP_{II} – water vapour permeability for experimental setup II

O_2P_I – oxygen permeability for experimental setup I

O_2P_{II} – oxygen permeability for experimental setup II

CO_2P_I – carbon dioxide permeability for experimental setup I

CO_2P_{II} – carbon dioxide permeability for experimental setup II

TS_I – tensile strength for experimental setup I

TS_{II} – tensile strength for experimental setup II

EB_I – elongation-at-break for experimental setup I

EB_{II} – elongation-at-break for experimental setup II

R_{O2} – O_2 consumption rate

R_{CO2} – CO_2 production rate

w – weight of the cheese

V_f – free volume of the container

E_{ax} – activation energy

T_{ref} – reference temperature (285 K)

T – temperature

R – universal gas constant (8.314×10^{-3} kJ mol^{-1} K^{-1})

T_g – glass transition temperature

PY – Polysaccharide yield

EY – extracts yield

T_m – peak melting temperature

V_0 – void volume

V_1 – total volume

F_n – oligosaccharide fractions for increasing molecular weights

TPC – total phenolic content

RSA – radical scavenging activity

DTG – derivate of the weight loss curve

Greek symbol

η – intrinsic viscosity

η_{rel} – relative viscosity

η_{sp} – specific viscosity

ΔE – total colour difference

γ_L^p – polar component of the liquid

γ_L^d – dispersive component of the liquid

γ_S^p – polar component of the surface

γ_S^d – dispersive component of the surface

θ – contact angle

γ_{SV} – solid-vapour interfacial tension

γ_{SL}– solid-liquid interfacial tension

γ_{LV} – liquid-vapour interfacial tension

ρ_{ch} – density of the cheese

ΔW – weight loss

ΔH_n – thermal transition

ΔH_m – enthalpy of melting

Abbreviations

RH – relative humidity

GRAS – generally recognized as safe

GT – *Gleditsia triacanthos*

CP – *Caesalpinia pulcherrima*

AP – *Adenanthera pavonina*

Poly - polysaccharide

Gly – glycerol

Sorb - sorbitol

CH – chitosan

NO – without coating

Man – mannose

Gal – galactose

Rha – rhamnose

Fuc – fucose

Ara – arabinose

Xyl – xylose

Glc – glucose

MS – mass spectrometry

ESI – electrospray ionization

TGA – thermogravimetric analysis

DSC – differential scanning calorimetry

FTIR – fourier transform infrared

SEM – Scanning electron microscopy

O_2 – oxygen

CO_2 – carbon dioxide

H_2SO_4 – sulphuric acid

H_2 – hydrogen

NaOH – sodium hydroxide

$NaBD_4$ – sodium tetradeuteroborate

H_2O – water

DPPH – 1,1-diphenyl-2-picrylhydrazyl

PMMA – poly(methyl methacrylate)

SS316 – stainless steel 316

PMAA – partially methylated alditol acetates

PTA – pre-treatment A

PTB – pre-treatment B

PTC – pre-treatment C

SEC – size-exclusion chromatography

BHT – 2,6-Di-tert-butyl-4-methylphenol

BHA – 3-ter-Butyl-4-hydroxyanisole

Na_2CO_3 – sodium carbonate

$Mg(NO_3)_2$ – magnesium nitrate

TPA – texture profile analysis

PCA – Plate Count Agar

PDA – Potato Dextrose Agar

HLB – hydrophilic/lipophilic balance

dHG – decolorized hsian-tsao leaf gum

PEG - polyethylene glycol

TPA – texture profile analysis

CHAPTER 1

MOTIVATION AND OUTLINE

The motivation and outline, and research aims of this work are approached in this chapter, where a general overview of the thesis is provided.

1.1 THESIS MOTIVATION

Food industry seeks for new and renewable products that lead to sustainability programs. In this context, food technology plays an important role, with main research areas such as chemistry, biochemistry, engineering and physics, seeking for new know-how and for the improvement of food quality during the processing, storage and handling. Today many researchers focus their efforts in the creation of new methodologies and materials that increase the shelf-life of foods, without affecting their quality (Rahman, 2007; Laurienzo et al., 2008).

Edible coatings and films are adding value to agricultural and food industries by-products. The increase on the food packaging market has been accompanied by a vast amount of knowledge on edible coatings and films, acquired through research and product development work, as well as in advances in material science and processing technology. Based on this, in the last years, a great interest has developed in the replacement of commercial synthetic plastics by renewable resources (Tharanathan, 2003; Han & Gennadios, 2005; Pavlath & Orts, 2009).

Cheese is the generic name for a group of fermented milk-based food products, being at the same time the most diverse group of dairy products. Cheese is biologically and biochemically dynamic and, consequently, unstable (Fox & McSweeney, 2004). Cheese protection with coatings and/or films of synthetic materials for moisture regulation and protection against contamination is a well-know procedure, using materials such as cellophane, cellophane-polyethylene, saran, parakote, pliofilm, cryovac, aluminium foil, aqueous dispersions of butyl rubber and a copolymer of vinyl and vinylidene chloride (Kampf & Nussinovitch, 2000).

The application of edible coatings and films has been hardly explored for dairy products. Many works have focused on the use of edible coatings and films to extend and improve the shelf-life of fruits and vegetables (Soliva-Fortuny & Martín-Belloso, 2003; Lin & Zhao, 2007). However, few works use edible coatings and films to extend the shelf-life of cheese (Kampf et al., 2000; Duan et al., 2007; Conte et al., 2009).

“Regional” cheese, used in this work, is a cylindrical, yellow and semi-hard cheese; it is sold with acetate polyvinyl coating with the addition of a commercial antifungal agent, and is subjected to a

great range of storage temperature conditions (0-20 °C), often suffering an excessive water loss. The replacement of this synthetic polymer by a biopolymer and the decrease of the water loss is one of the goals of the industry at this moment.

1.2 RESEARCH AIMS

The main objective of this thesis was the development and characterization of new edible coatings for cheese preservation. Figure 1-1 shows a flow chart of the outline and the main ideas of this thesis. The main focus areas were:

- Physicochemical characterization of new galactomannan sources;
- Evaluation of the incorporation of phenolics and antioxidants compounds on edible films;
- Study of the influence of plasticizer and corn oil on edible coatings/films properties;
- Selection of the best coatings/films for cheese preservation based on their properties;
- Study of the influence of coatings and temperature in cheese gas exchange;
- Study of the coating application and temperature in cheese physicochemical properties and evaluation of application method in coatings effectiveness.

1.3 THESIS OUTLINE

The thesis is organized in eight chapters. In this chapter the motivation, research aims and the thesis outline are described. Chapter 2 presents an overview on the utilization of renewable sources, with a great focus in polysaccharide materials, for the production of edible coatings/films, their properties and applications. For Chapters 3 to 7 contain the main experimental results, distributed as follows:

Chapter 3 presents a methodology for the extraction of galactomannans from seeds of three different species of *Leguminosae* (*Gleditsia triacanthos*, *Caesalpinia pulcherrima* and *Adenanthera pavonina*). The yields of extraction and physicochemical properties of the obtained galactomannans were discussed and compared with commercial galactomannans.

In Chapter 4 three different extraction procedures were performed to obtain galactomannans from *G. triacanthos* seeds. Ethanolic and aqueous extracts were obtain and characterized in terms of the yield of extraction, total phenolic content and antioxidant capacity. Different concentrations of the extracts were incorporated into *G. triacanthos* galactomannan solutions and films properties were evaluated.

Chapter 5 shows the influence of glycerol and corn oil on chitosan and galactomannan coatings/films properties. The effects of glycerol and corn oil on properties such as wettability, moisture content, solubility, water vapour permeability, mechanical properties and opacity were discussed, supported by Fourier transform infrared spectroscopy, differential scanning calorimetry and thermogravimetric analyses.

Chapter 6 explores the ability of chitosan and galactomannans to be used as coatings for cheese. Chitosan and galactomannan coatings/films, with different formulations, were evaluated based on their properties. The best formulation for each polysaccharide coating forming solution was chosen based on their wettability on cheese, and then on their transport properties, as well opacity and mechanical properties.

Chapter 7 determines the influence of the application of chitosan and galactomannan coatings and of storage temperature on the gas exchange rate of "Regional" cheese. The coating that showed the greatest influence on the cheese gas exchange, decreasing O_2 consumption and CO_2 production rates was applied on cheese. Shelf-life parameters were monitored through the performance of chemical and microbiological analyses. The selected coating was subsequently applied by different application methods being the effectiveness of each coating method discussed.

Finally, Chapter 8 presents the overall conclusions, recommendations and suggestions for future work.

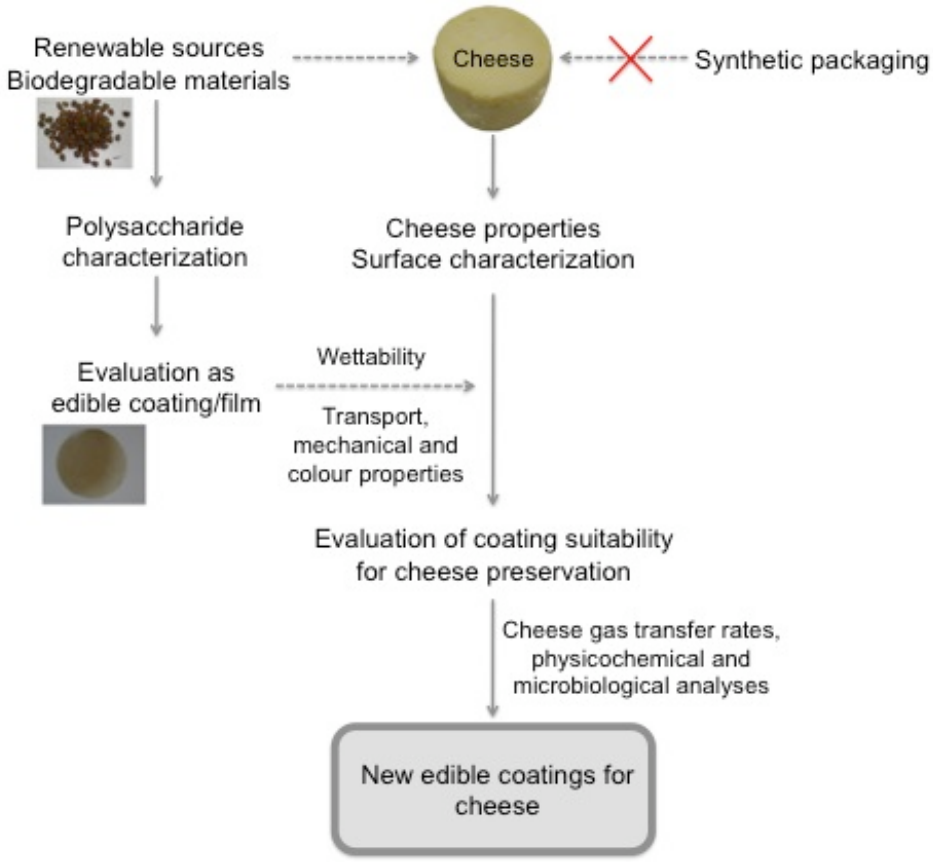

Figure 1-1. Flow chart of the outline and the main ideas of this thesis.

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CHAPTER 2

HYDROCOLLOID BASED EDIBLE COATINGS/FILMS FOR FOOD APPLICATIONS

This chapter provides a general overview of the materials used for the production of edible coatings and films, with a greater focus on polysaccharides, their main physicochemical properties and potential applications.

2.1 INTRODUCTION

Conventional synthetic packaging films had led to serious ecological problems due to their non-biodegradability. In this context, biopolymers can be an alternative source for packaging development (Siracusa et al., 2008). In the last decade, there has been a growing interest in the development of thermoplastic materials from biodegradable biopolymers, particularly those derived from renewable resources (Petersen et al., 1999). Biopolymers have been slow to reach commercial maturity, due to the higher costs and less optimal physical properties when compared with synthetic plastics. In addition, there have not been sufficient incentives for downstream processors to incorporate the biodegradable materials into their products (Siracusa et al., 2008). The ecological impact of raw material resources used in manufacturing products and their ultimate disposal are relevant considerations in their design. Products designated, as "ecoefficient" are the new generation of bio-based products made from sustainable materials that conform to ecological and economic requirements (Narayan, 1994; Narayan, 1998). Biobased packaging is defined as packaging containing raw materials from agricultural sources, i.e. produced from renewable biological raw materials. To date, biodegradable packaging has attracted great attention, and numerous projects are under way in this field. One important reason for this attention is the marketing of environmentally friendly packaging materials. Furthermore, the use of biodegradable packaging materials has the greatest potential in countries where landfill is the main waste management tool (Petersen et al., 1999; Farris et al., 2009; Mahalik & Nambiar, 2010).

The edibility of the materials used for coatings/films production can also be an issue, in order to consider the final materials as "edible". One of the great problems is the solvent used; so in order to maintain their edibility only water and ethanol should be used (Han et al., 2005). These materials used must have to be approved as GRAS (generally recognized as safe). However, GRAS status does not guarantee complete product safety, especially for consumers who have food allergies or sensitivities, such as lactose intolerance (milk) and Celiac disease (wheat gluten). In general the GRAS status should be attached to the used materials to the production of edible coatings/films (Pavlath et al., 2009).

Edible coatings/films are two terms used in food packaging, sometimes without any distinction. However, it is important to make the distinction: the "film" is a thin skin formed by the casting of the biopolymer solution prepared separately from the food that is later applied to it, while the "coating" can be a suspension or an emulsion applied directly on the surface of the food, leading to the subsequent formation of a film (Krochta, 2002).

The use of edible coatings/films based on natural polymers and food grade additives has been constantly increasing in the food industry. The utilization of edible coatings/films to extend food shelf-life has been highlighted based on the need for high quality and the demand for minimal food processing and storage technologies. They can be used to regulate the transfer of moisture, oxygen, carbon dioxide, aroma, and taste compounds in a food. Food products are usually coated by dipping or spraying, forming a thin film on the food surface, that acts as a semipermeable membrane, that can control the moisture loss or/and suppress the gas transfer (Lin & Zhao, 2007).

Edible coatings/films can be utilized for most foods to meet challenges associated with stable quality, market safety, nutritional value, and economic production cost. The main and potential advantages or/and benefits of using edible coatings/films are (Figure 2-1): to provide a moisture barrier (decreasing the problems associated to moisture loss); to provide a gas barrier for controlling gas exchange between the food and the surrounding atmosphere (allowing the slow down of respiration and delaying deterioration); to restrict the exchange of volatile compounds (preventing the loss of natural volatile flavour compounds and colour components); to protect from physical damage of produce caused by mechanical impact, pressure, vibrations, and other mechanical factors; to act as carriers of other functional ingredients, such as antimicrobial and antioxidant agents (Lin & Zhao, 2007).

2.2 MATERIAL USED IN EDIBLE COATINGS/FILMS

Coatings/films can be produced with a great variety of products such as polysaccharides, proteins, lipids, resins, with the addition of plasticizers and surfactants. Polysaccharides that have been used to form films coatings include starch and derivatives, cellulose and derivatives,

alginates, carrageenan, various plant and microbial gums, chitosan, and pectinates (Lin & Zhao, 2007; Rinaudo, 2008). Their hydrophilic properties provide a good barrier to CO_2 and O_2 under certain conditions but a poor barrier to water vapour and deficient mechanical properties (Guilbert, 1986; Park, 1999).

The functionality and performance of edible coatings/films mainly depends on their barrier and mechanical properties, which in turn depend on film composition, its formation process and the method of application on the product.

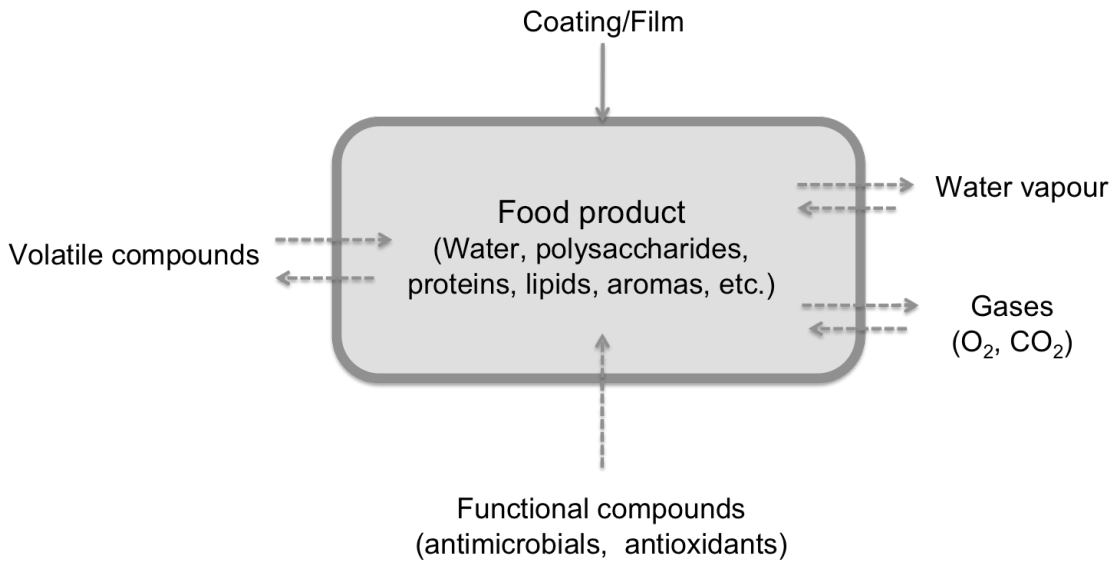

Figure 2-1. Functional properties of edible coatings/films on food products (Adapted from Lin & Zhao, 2007).

2.2.1 Polysaccharides

Polysaccharides are natural polymers composed of monosaccharide residues that are connected by O-glycosidic linkages, and depending on the source can be neutral, positively or negatively charged. They either act as energy-rich food stores in plants and animals, or have structural roles in the plant cell wall or the tough outer skeleton of insects and other animals (Nelson & Cox, 2000). The great diversity of structural features of polysaccharides have origin from differences in the monosaccharide composition, linkage types and patterns, chain shapes, and degree of

polymerization, influencing their physical properties as: solubility, gelling potential, and/or surface and interfacial properties. Polysaccharides, which are commercially available for use in food and nonfood industries as stabilizers, thickening and gelling agents, crystalization inhibitors, and encapsulating agents, etc., are also called gums. Gums are a group of industrially useful polysaccharides or their derivatives that hydrate in hot or cold water forming viscous solutions or dispersions at low concentrations. When used in foods, gums are sometimes referred as hydrocolloids, and they are classified as natural and modified gums. Natural gums include seaweed extracts (e.g. alginates), plant exudates (e.g. arabic and tragacanth gums), gums from seeds or roots (e.g. galactomannan and potato starch), and gums obtained by microbial fermentation (e.g. xanthan) (Izydorczyk et al., 2005; Stephen & Churms, 2006).

Two polysaccharides were studied in the present work as materials for the production of edible coatings/films; chitosan, due its full characterization and known applications in the food industry, and galactomannans, also greatly applied in food industry as thickener, stabilizer and emulsifier agents, but unexplored as coating. The most important characteristics of these two polysaccharides are presented below.

2.2.1.1 Chitosan

Chitin is the principal component of the hard exoskeletons of nearly a million species of arthropod insects and crustaceous (e.g. lobsters, and crabs); and is probably the second most abundant polysaccharide in nature, next to cellulose. It is a linear homopolysaccharide composed of N-acetylglucosamine residues in β-linkage. Chitin forms extended fibers similar to those of cellulose, and like cellulose cannot be digested by vertebrates (Kumar, 2000). The deacetylation process can convert chitin into another form of polyaminosaccharide, called chitosan. Chitin is a common group of solid waste (e.g. from films processing industries) in many countries, and the utilization of chitosan would provide benefits of minimizing solid waste.

Chitin can be obtained from crab or shrimp shells or from fungal mycelia. For the first case the achievement is associated with food industries such as shrimp canning where the process involves the proteins removal and the dissolution of calcium carbonate that is present in crab shells at high concentration. The obtained chitin is then deacetylated in sodium hydroxide at high

temperatures obtaining the deacetylated chitosan (Figure 2-2a). In the second case the production of the chitosan-glucan complexes is associated with a fermentation process (*Aspergillus niger*, *Mucor rouxii* and *Streptomyces*) that involves an alkali treatment, where depending on the alkali concentration some glycans are removed.

There are three types of reactive functional groups in chitosan: an amino group as well as primary and secondary hydroxyl groups at C-2, C-3, and C-6 positions, respectively (Shahidi et al., 1999). This chemical structure makes chitosan easily modified as immobilization support compared to other materials. Both chitin and chitosan could serve as material for making carriers for enzymes and cells (Krastanov & Yoshida, 2003). Chitosan has several important advantages such as biocompatibility, biodegradability and no toxicity; and several studies indicated chitosan as bacteriostatic and fungistatic (Kumar, 2000; Dutta et al., 2009). Because of their nontoxicity and biocompatibility, the application of chitin and chitosan-based materials covers not only food environmental systems, but also, pharmaceutical, medical, and agricultural industries (Prashanth & Tharanathan, 2007).

The polycationic properties of chitosan provide the possibility of film formation by the breakage of polymer segments and subsequent reforming of the polymer chain into a film matrix or gel; this can be achieved through the evaporation of the solvent thus creating hydrophilic and hydrogen bonding and/or electrolytic and ionic crosslinking. Furthermore, chitosan films are tough, flexible, and very difficult to tear. Most mechanical properties of chitosan films are comparable to those of many medium-strength commercial polymers (Butler et al., 1996; Aider, 2010).

2.2.1.2 Galactomannan

Galactomannans are polysaccharides built up of a β-(1–4)-D-mannan backbone with a single D-galactose branches linked α-(1–6) (Figure 2-2b). They are present in the endosperm of numerous plants, particularly the *Leguminosae*, and have several functions. The endosperm serves as a food reserve for germinating seeds and it retains water, preventing the complete drying of seeds (Srivastava & Kapoor, 2005; Gidley & Reid, 2006).

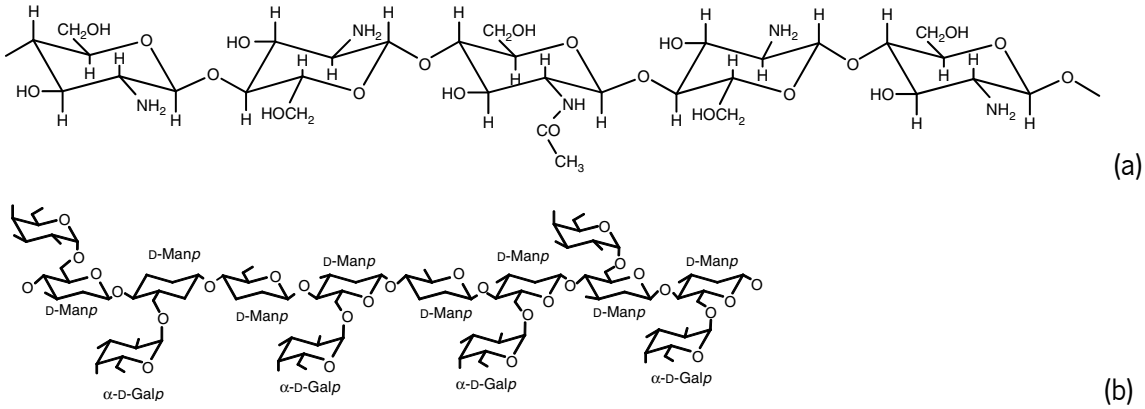

Figure 2-2. Chemical structure of chitosan (a) and galactomannan (b) polysaccharides (Adapted from Izydorczyk et al., 2005).

Galactomannans can often be used in different forms for human consumption. Featuring different physicochemical properties, galactomannans are a versatile material used for many applications: they are excellent stiffeners and stabilizers of emulsions, and the absence of toxicity allows their use in the textile, pharmaceutical, biomedical, cosmetics and food industries (Baveja et al., 1991; Krishnaiah et al., 2002; Varshosaz et al., 2006; Vendruscolo et al., 2009; Vieira et al., 2007). The principal applications of galactomannans in food industry are in dairy products, fruit-based water gels, powdered products, bakery, dietary products, coffee whiteners, baby milk formulations, seasonings, sauces and soups, tinned meats, and frozen and cured meat foods. This broad range of applications reflects a number of different functional characteristics including high solution viscosity (guar), stabilization of frozen systems (guar and locust bean gum), and mixed gel formation with other polysaccharides and proteins (locust bean gum) (Gidley & Reid, 2006). Some works showed the possibility of using galactomannans in the formation of films (Aydinli & Tutas, 2000; Mikkonen et al., 2007).

The process to obtain galactomannans from seeds combines the extraction and purification of the polysaccharides from the endosperm. Briefly, the seed hull is removed from the seeds, and the germ is separated from the endosperm. The endosperm is dissolved in water (cold or hot water) and the galactomannan is obtained upon precipitation with alcohol (Dakia et al., 2008; Cunha et al., 2009; Vendruscolo et al., 2009).

The degree of substitution in galactomannans profoundly affects their solution properties, and differs according to the species (Neukom, 1989; Kök et al., 1999; Izydorczyk et al., 2005). The

characterization of galactomannans' structure can be performed using different techniques. The most important parameters that define the nature of a gum are the mannose/galactose (*M/G*) ratio, the average molecular weight, the fine structure, and the intrinsic viscosity. The monomeric sugars content and *M/G* ratio are generally determined by gas chromatography or by high-pressure anion exchange chromatography after partial or total hydrolysis catalyzed by acid. The molecular weight distributions can be determined by size exclusion chromatography. The galactose repartion along the mannane chain can be characterized by ^{13}C NMR spectroscopy, or by enzymatic methods with β-D-mannanase that degrade specifically the nonsubstituted regions of galactomannans (Dakia et al., 2008; Cunha et al., 2009; Vendruscolo et al., 2009). Intrinsic viscosity can be determined using a capillary viscometer, by application of Huggins' and Kramer's equations. Furthermore, the evaluation of rheological behaviour play an important role in the characterization of galactomannans solutions, since they are often used to modify textural attributes (Marcotte et al., 2001). This characterization can be performed through shear (steady and dynamic conditions) and extensional rheology (Bourbon et al., 2010).

The three major galactomannans of commercial importance in food and non-food industries are guar gum (GG, *Cyamopsis tetragonolobo*, *M/G* ratio: 2:1), tara gum (TG, *Caesalpinia spinosa*, *M/G* ratio: 3:1) and locust bean gum (LBG, *Ceratonia siliqua*, *M/G* ratio: 3.5:1 (Gidley & Reid., 2006; Dakia et al., 2008). However, the industry trends demand the introduction of alternative sources of seed gums and it is therefore important to search for alternative renewable sources (Joshi & Kapoor, 2003). The introduction of alternative sources of galactomannans requires their characterization, which may be performed using the techniques mentioned above.

2.2.2 Proteins

Proteins cover a broad range of polymeric compounds that provide structure and biological activity in animal and plants. They are heteropolymers comprised of more than 20 different amino acids, and have specific sequences and structures. All the common amino acids have a carboxyl group (COOH) and an amino group (NH_3) bound to a carbon atom. They differ from each other by their side chain group that varies in structure, size, electric charge, and that influence

the solubility in water. This side chain group imparts a unique character to the amino acid and can be non-polar, polar, uncharged, aromatic and positively or negatively charged (Cheftel et al., 1985; Nelson & Cox, 2000).

Proteins are characterized by their primary, secondary and tertiary structures. The sequential order of the amino acids corresponds to the primary structure. The backbone structure along the polymer chain, based on Van der Waals, hydrogen bonding, electrostatic, hydrophobic, and disulfide cross-link interactions among the amino acid units corresponds to the secondary structure. The tertiary structure reflects the secondary structure organization relative to each other, forming generally globular, fibrous, or random structures (Cheftel et al., 1985; Nelson & Cox, 2000; Rose, 2004). Protein structure (secondary and tertiary structures) can be modified by various physical and chemical agents, including heat, mechanical treatment, pressure, irradiation, lipid interfaces, acids and alkalis, and metal ions (Cheftel et al., 1985).

Due to this molecular diversity, proteins have considerable potential for the formation of linkages that differ with respect to their position, nature, and/or energy. Proteins can be divided into proteins from plant origin (e.g. gluten, soy, pea and potato) and proteins from animal origin (e.g. casein, whey, collagen, and keratin). The side chains of these amino acids are highly suitable to chemical modification which is helpful to the material engineer when tailoring the required properties of the packaging material (Nelson & Cox, 2000).

Protein film-forming materials derived from animal sources include collagen, gelatin, fish myofibrillar protein, keratin, egg white protein, casein, and whey protein. Protein film-forming materials derived from plant sources include corn zein, wheat gluten, soy protein, peanut protein, and cottonseed protein. Proteins can be good film formers and may be used in coating formulations for fruits and vegetables. Protein films are effective as gas barriers (O_2 and CO_2) but their water vapour transmission rates are high (Krochta, 2002).

2.2.3 Plasticizer, surfactant and other biopolymers

The formation of coatings/films from polysaccharides requires in the most cases the presence of a plasticizer. The films without plasticizer present a brittle and stiff structure due to the extensive interactions between polymer molecules. So normally, plasticizers are added to the film in order to improve their physical properties (Bergo & Sobral, 2007). They help decreasing brittleness and improving flexibility, through reducing the intermolecular forces and the increase of the mobility of polymeric chains (Sothornvit & Krochta, 2001; Rivero et al., 2010). Most plasticizers are very hydrophilic and hygroscopic. The most usual plasticizers are polyols, mono-, di-, and oligosaccharides. Polyols have shown to be particularity effective to plasticize hydrophilic hydrocolloids. Glycerol is the most used, but also sorbitol, ethylene glycol, and sucrose were tested as plasticizers (Cherian et al., 1995; Cuq et al., 1997; Hernandez-Munõz et al., 2004; Bergo & Sobral, 2007). Glycerol is a major by-product of biodiesel production, which has significantly increased, thus creating a glycerol surplus. In the past, the biodiesel industry considered glycerol a desirable co-product that could contribute to the economic viability of biodiesel production; however, nowadays, glycerol is often regarded as a waste stream with an associated cost (Fountoulakis & Manios, 2009). The use of glycerol as plasticizer in coatings/films can be a way to help solving the existing surplus of this co-product from biodiesel production.

Surfactants are amphoteric substances due to their simultaneous hydrophilicity and hydrophobicity properties, and are conventionally added to enhance the stability of the emulsion. They can be incorporated into the coating to reduce the surface tension of the solution, improving the wettability of the coatings (Choi et al., 2002; Ribeiro et al., 2007; Ziani et al., 2008).

The hydrophilic behaviour of polysaccharides is responsible for the lower water vapour permeability (*WVP*) of polysaccharide coatings/films. The decrease of their hydrophilic behaviour has been studied in the last years; different materials (lipids, surfactant, rigid particles, fibers, etc) were tested with the main objective of decreasing *WVP* (Garcia et al., 2000; Morillon et al., 2002; Ziani et al., 2008; Casariego et al., 2009; Chen et al., 2009).

The incorporation of active substances as antimicrobials, antifungals, and antioxidants are one of the emerging utilizations of edible coatings/films. However, this incorporation can also lead to the change of the physicochemical properties of edible films (Gómez-Estaca et al., 2009; Gemili et al., 2010)

2.3 COATINGS AND FILMS CHARACTERIZATION

2.3.1 Wettability

Wettability can be used as a measurement of the effectiveness of edible coatings to coat a food surface (Park, 1999). Coatings must wet and spread on the surface of the food product, and form a film upon drying that has the adequate properties and durability. The coating process involves wetting of the food product by the coating solution, and the possible penetration of the solution into the skin (Hershko et al., 1996). The wettability of a solid by a liquid is determined by the balance between adhesive forces of the liquid on the solid and cohesive forces of the liquid, where adhesive forces cause the liquid to spread over the solid surface while cohesive forces cause it to shrink. Wettability is one of the most important properties when evaluating the capacity of a solution to coat a designated surface. In practical terms, the closer the W_s values are to zero, the better a surface will be coated (Ribeiro et al., 2007). Surface energy or surface tension of the food product is also a controlling factor in the process that involves the coating application (Karbowiak et al., 2006). The determination of the surface tension usually involves the measurement of the contact angles that several standard liquids make with that surface. The surface energy of the solid surface is then related to the surface tensions of the liquids and the contact angles. This method invokes an estimation of the critical surface tension of the surface of the solids studied, by extrapolation from the Zisman plot (Zisman, 1964).

Some studies evaluate the wettability of coating solutions on the fruit and/or vegetable to be coated. Choi et al. (2002) studied the wettability of chitosan coating solutions on 'Fuji' apple skin using the Du Nouy ring method and the sessile-drop method. The 'Fuji' apple skin surface presented a critical tension of 18.7 mN m^{-1} (Choi et al., 2002). Ribeiro et al. (2007) obtained a similar value for the critical surface tension of strawberries (18.84 mN m^{-1}), which were reported

to have a superficial tension of 28.94 mN m^{-1}, with polar and dispersive components of 5.95 and 22.99 mN m^{-1}, respectively. In both cases, to enhance the wettability of the coating solutions, Tween 80 was added, reducing the superficial tension of the liquid and thus increasing the spreading coefficient (W_s) (Ribeiro et al., 2007). Casariego et al. (2008) determined the effects of the concentrations of plasticizers, Tween 80 and chitosan on the wettability of chitosan-based edible coatings in view of their application on tomato and carrot. They present the superficial tensions of tomato and carrot as being 28.71 and 26.48 mN m^{-1}, respectively. The increase of chitosan concentration and the presence of glycerol or sorbitol as plasticizers decreased the values of W_s and adhesion coefficients; being the best experimental values of W_s obtained for the coating composition of 1.5 % (w/v) of chitosan and 0.1 % (w/w) of Tween 80 (Casariego et al., 2008). A good choice of the coating formulation is essential for the durability and maintenance of the coating on the food products. The determination of wettability, with the study of the W_s, work of adhesion and cohesion, as well as the study of the surface properties of the products, is therefore fundamental for the correct application of edible coatings.

2.3.2 Transport properties

Edible coatings/films can provide a combination of moisture, oxygen, carbon dioxide, flavour, aroma, colour, or oil barrier for a food or drug, with a resulting increase in quality and shelf-life of food products. Thus, the permeability of edible films to these substances is of interest.

Permeability is a steady-state property that describes the extent to which a permeating substance dissolves and then the rate at which the permeant diffuses through a film, with a driving force related to the difference in concentration of the permeate between the two sides of the film (Krochta, 2002). To better understand the permeability properties some film characteristics are important, such as the polymer structure, the degree of polarity (cohesive energy density), interstitial space between the polymer molecules (free volume), polymer chain mobility (cristallinity), alignment of the polymer chains and in the polymer backbone (orientation) (Miller & Krochta, 1997). The transport properties most studied in edible coatings/films are the

permeability to water vapour (*WVP*), oxygen (O_2P), carbon dioxide (CO_2P), and to aromas and flavours.

A great amount of studies were done on the effects of different factors in *WVP*, O_2P and CO_2P of edible films (Bangyekan et al., 2006; Bergo & Sobral, 2007; Carneiro-da-Cunha et al., 2009; Casariego et al., 2009; Chen et al., 2009). The effects of acid and plasticizer concentrations have been studied in chitosan-based films. Results showed that higher concentrations of plasticizer lead to higher permeability values. There were explained by the plasticizer effect, that decreases the intermolecular attractions between polymeric chains, facilitating the penetration of gas molecules (Caner et al., 1998). More recently, studies of O_2P and *WVP* of chitosan films with different plasticizers (glycerol, sorbitol and polyethylene glycol (PEG)) and fatty acids (stearic and palmitic acids), showed that the addition of glycerol and sorbitol into chitosan films increased O_2P, whereas the addition of PEG decreased O_2P when compared with that of chitosan films without plasticizer. Moreover, results showed that the addition of plasticizer to chitosan films lead to a decrease of the *WVP* values, being the presence of glycerol responsible for the lowest values while PEG originated the highest values (Srinivasa et al., 2007). The same behaviour was observed for starch-alginate films where *WVP* values decreased with the addition of glycerol (Maizura et al., 2007). Studies with calcium-alginate films showed that there was no difference in *WVP* between glycerol-free and glycerol-containing films, while the films with fructose and sorbitol showed the lowest *WVP* values and those containing PEG showed the highest values (Olivas & Barbosa-Cánovas, 2008). In another work, the effect of a plasticizer and a surfactant in the *WVP* of chitosan films was studied with the addition of glycerol and Tween 20. Results showed that a positive interaction exists between glycerol and Tween 20, with an increase of *WVP* for higher concentrations of glycerol and Tween 20. Also on films where no Tween 20 was added, the increase of glycerol concentration leads to an increase *WVP*. Only for those cases where chitosan films were made without glycerol the presence of Tween 20 did not have a significant effect on *WVP* (Ziani et al., 2008). The water barrier properties of starch/decolorized hsian-tsao leaf gum films are significantly influenced by surfactants: the increase of the hydrophilic/lipophilic balance of the surfactant leads to a decrease of *WVP*. Moreover, *WVP* also decreased when the surfactant content increased in the film composition (Chen et al., 2009). Results obtained for starch-based films showed that the absence of plasticizer in starch films leads to an increase of O_2P and CO_2P,

attributed to the presence of cracks and pores in the unplasticized films. Moreover, films containing sorbitol presented the lowest values of O_2P and CO_2P (Garcia et al., 2000). The *WVP* of galactomannan (LBG) based films was studied for increasing polyethylene glycol (PEG) concentrations with different molecular weights (Aydinli & Tutas, 2000). Galactomannan films with the addition of PEG (of molecular weight between 200 and 600) showed that *WVP* values increase for higher concentrations of plasticizer. However, when PEG 1000 was used the *WVP* values decreased when higher concentrations of plasticizer were added. The different behaviour between the PEG 1000 and the PEG with lower molecular weight has been explained by the solid state of the PEG 1000 at room temperature. Moreover, PEG 200 presents the lowest values of *WVP* due to its lower hydrophobic character when compared with the PEG of higher molecular weight.

Lipids, due their hydrophobic behaviour, are added to polysaccharide films aiming at decreasing their hydrophilicity, consequently decreasing the values of *WVP*. In a recent work, the addition of oleic acid to chitosan films has shown to decrease *WVP* values (Vargas et al., 2009). The addition of lipids was also tested in starch-based films. Results have shown that the addition of sunflower oil decreases the *WVP* of such films (Garcia et al., 2000). Similar results were obtained for corn starch and methylcellulose films where the use of soybean oil in led to a decrease of *WVP* values (Bravin et al., 2006). Also arabinoxylan films with hydrogenated palm oil displayed lower *WVP* values than films without oil (Péroval et al., 2002). The effects of different lipids (beeswax, stearic-palmitic acids) addition were studied in gellan films. Results demonstrated that lipids addition to gellan films decreased *WVP*, being beeswax more effective than stearic-palmitic acids (Yang & Paulson, 2000). On the contrary, the addition of saturated fatty acids (palmitic and stearic acid) to chitosan films showed not to affect the *WVP* values (Srinivasa et al., 2007). In another work, LBG galactomannan was blended with two lipids and the *WVP* of the resulting film was evaluated. Results showed that films with stearoptene and beeswax presented lower *WVP* values that control films. Moreover it has been shown that when using PEG 200 and sorbitol as plasticizer the LBG films with stearoptene presented lower *WVP* values that LBG films with beeswax, these results being explained by the higher hydrophilic behaviour of beeswax (Bozdemir & Tutas, 2003).

Protein films present in some way the same behaviour of polysaccharide films when plasticizers and oil are added to the film matrix. Moreover, the addition of polysaccharides can also be used to improve the transport properties of protein films. Despite the fact that their hydrophilic character does not allow them to be as efficient as lipids in terms of water vapour permeability, generally the blending of polysaccharides with proteins is easier to perform. The addition of pullulan to whey protein isolate (WPI) resulted successfully in decreasing the *WVP* values of protein films (Gounga et al., 2007). The addition of potato starch and sodium alginate to WPI improved the transport properties of this kind of protein films (Ciesla et al., 2006). The addition of cellulose to soy protein isolate (SPI) films was tested and results showed that the increase of the cellulose concentration leads to a decrease of *WVP* values (Wu et al., 2009).

The incorporation of active substances such as antimicrobials, antifungals, and antioxidants is one of the emerging utilizations of edible coatings/films. This incorporation can lead to changes of the physicochemical properties of edible films (Lee, 2005). For starch-alginate films the *WVP* of films increased significantly with the addition of lemongrass oil. The lemongrass oil causes an increase of flexibility in the polymeric structure and contributes to the increase of water absorption by the film (Maizura et al., 2007). The effects of ferulic acid on the barrier properties of starch-chitosan blended films were studied and the results showed that for those films the incorporation of oxidized ferulic acid improved considerably the barrier properties of the films. Ferulic acid acts as a cross-linking agent in starch-chitosan blended films leading to a decrease of the oxygen transmission rate of the resulting films (Mathew & Abraham, 2008).

2.3.3 Thermal and mechanical properties

Edible films with satisfactory mechanical properties and good appearance are potential and ecological alternatives for substituting synthetic packaging in e.g. pharmaceutical and food applications. The most used mechanical tests applied on commercial films are also used to characterize edible coatings/films. They are: tensile strength (*TS*), elongation-at-break (*EB*), Young's modulus and puncture strength. Many times the mechanical properties of edible films are related with the glass transition temperature (T_g), but they can also be used to explain

transport properties. T_g is one of the most important parameters when determining the mechanical properties of amorphous and semicrystalline materials and in controlling the recrystallization process. The T_g is exhibited by amorphous polymers or amorphous regions of partially crystalline polymers when a viscous or rubbery state is transformed into a hard, brittle, glass-like state (Hatakeyama & Quinn, 1999).

Many authors have studied thermal and mechanical properties of edible coatings/films as a function of polymer structure, plasticizer content, storage relative humidity, antimicrobial incorporation, and in the presence of lipids.

The influence of the relative humidity (RH) and type and concentration of plasticizer in mechanical properties of alginate films were studied by several authors. Results showed that, in general, the increase of RH leads to a decrease of *TS* and an increase of *EB* for all films, acting the water content as a plasticizer in hydrophilic films. The addition of a plasticizer to alginate films leads to an increase of the capacity to adsorb water, thus justifying the great influence of RH in the films' mechanical properties (Olivas & Barbosa-Cánovas, 2008). Corn, cassava and yam starch films were evaluated for their thermal, mechanical and barrier properties. Results showed that when a plasticizer (glycerol) was incorporated into the starch network the values of T_g decrease when compared with those of unplasticized films. *TS* and Young's modulus decreased and *EB* increased with the increase of glycerol concentration (Mali et al., 2006). Mechanical properties of chitosan films were evaluated for different polyols (glycerol, sorbitol and PEG) and fatty acids (stearic and palmitic acids). Results showed that *TS* decreased with the addition of polyols and fatty acids, while *EB* increased with the addition of polyols (Srinivasa et al., 2007). The degree of deacetylation of chitosan is one of the most important structural characteristics of this polymer and has been related with its mechanical properties. It has been shown that chitosan films with a lower degree of deacetylation have higher *TS* and *EB* values (Ziani et al., 2008). However, other similar works reported that chitosan films with higher degree of deacetylation have a higher *TS* than those with a lower degree of deacetylation, explained by the higher crystallinity of the former (Chen et al., 1994). Also the structure of galactomannans can influence the mechanical and thermal properties of films. Galactomannans with lower content on galactose produced films with higher *EB* and *TS*, being the mechanical properties of films improved by the decrease of the degree of polymerization. These results suggest that the

removal of side groups might facilitate the sliding of polymers across each other, thereby increasing the *EB* values of the films, while the increase in *TS* could be due to more dense packing of mannan chains, resulting in increased hydrogen bonding (Mikkonen et al., 2007).

The influence of sucrose ester surfactants with different hydrophilic/lipophilic balance (HLB) values was evaluated on mechanical properties of tapioca starch/decolorized hsian-tsao leaf gum (dHG) films. It has been shown that the increase of HLB values of the surfactant the *TS* of starch/dHG/surfactant composite films decreased, and when the surfactant content was increased the *TS* and *EB* decreased (Chen et al., 2009).

The incorporation of antimicrobials can have a substantial effect on coatings/films properties, as those compounds may act as plasticizers or increase their crystallinity. Films of a mixture of partially hydrolyzed sago starch and alginate were produced with the addition of lemongrass oil and glycerol as natural antimicrobial agent and plasticizer, respectively. In the absence of glycerol, the *TS* of the film decreased as the oil content increased while the percent *EB* values for a film with 20 % glycerol were found to increase significantly with the increasing of lemongrass oil content.

2.3.4 Opacity and colour

The opacity of a material is an indication of how much light passes through it and can thus be important to control the incidence of light on a given food product. The higher the opacity, the lower the amount of light that can pass through the material. Also, film colour can be an important factor in terms of consumer acceptance. In the $L^* a^* b^*$ colour system, L^* represents the lightness, and a^* and b^* are colour coordinates, where $+a^*$ is in the red direction, $-a^*$ is in the green direction; $+b^*$ is in the yellow direction, $-b^*$ is in the blue direction, low L^* is dark, and high L^* is light. Also the whiteness index, the yellowness index, and the total colour difference (ΔE) are commonly used to characterize films.

It has been reported that for chitosan films L^* coordinates are normally higher than 97, and any added component will affect this colour parameter. However, the addition of Tween 20 makes

the films yellower (thus increasing the *b** parameter), which could be attributed to the original colour of Tween 20 (Ziani et al., 2008). Casariego et al. (2009) obtained opacity values of 8.23 % for chitosan films, and showed that such values are not affected by the increase of chitosan concentration or by the incorporation of micro/nano clay (within the limits of the study performed). They also showed that an increase of chitosan concentration tends to decrease the values of *L** and *a**, increasing the *b** values (this parameter describes the natural yellowish colour of chitosan) (Casariego et al., 2009). Also for chitosan films it has been demonstrated that the whiteness index values were significantly affected by the oleic acid content. The addition of oleic acid promoted a significant decrease in the whiteness index of the composite films (compared with chitosan film alone) (Vargas et al., 2009).

The influence of glycerol and chitosan on tapioca starch edible films was studied; and results showed that the yellow index, *b**, and *L** increase with chitosan concentration, while *a** is affected by glycerol concentration (Chillo et al., 2008). For tapioca starch/decolorized hsian-tsao leaf gum films, it has been shown that opacity increased with the addition of a surfactant and beeswax (Chen et al., 2009).

2.3.5 Water solubility

The solubility of an edible material indicates its integrity in an aqueous environment and can indicate its water resistance. Film solubility is an important factor that determines biodegradability of films when used as packaging materials (Gnanasambadam et al., 1997). If the films are intended for use in preservation of intermediate or high moisture foods, and/or incorporate antimicrobial compounds, low water solubility values are required. An antimicrobial film with poor water resistance will dissolve quickly, causing losses of the antimicrobial agent (Ozdemir & Floros, 2008). The solubility of biopolymers is highly influenced by their structure and by the constituents that form the film's matrix.

Solubility of films from three different polysaccharides was tested by Tong et al. (2008). Results showed that pullulan films dissolved much faster in water than alginate and carboxymethylcellulose films. It has also been shown that the solubility of starch-alginate based

films increases with the addition of glycerol (Maizura et al., 2007). The hydroxyl groups of glycerol increase the hydrophilicity of films thus increasing their solubility. Also the solubilization temperature can be an influencing factor in the solubility of films. For corn starch and chitosan films the solubility increases in water at increasing temperatures (Garcia et al., 2006). The polysaccharide's structure has been shown to be the most important factor affecting the solubility of films; however, the presence of hydrophilic or hydrophobic compounds can also influence the values of water solubility of edible films.

2.4 INCORPORATION OF ACTIVE COMPOUNDS IN EDIBLE COATINGS/FILMS

Edible coatings/films have the capability to act as carriers for a wide range of food additives, including antioxidants, antibrowning agents, various antimicrobials, colorants, and flavours that can extend product shelf-life and reduce the risk of pathogen growth on food surfaces and enhance the sensory quality of wrapped or coated food (Vargas et al., 2008). The interactions with the food or the food environment make of edible coatings/films an excellent alternative to traditional food packaging. Several studies were carried out addressing the properties that some compounds have as antimicrobial and antioxidant agents such as plant extracts (oregano, rosemary), enzymes (lysozyme), bacteriocins (nisin) and salts (potassium sorbate) (Cagri et al., 2004; Pranoto et al., 2005; Gómez-Estaca et al., 2009).

The selection of incorporated active agents should be limited to edible, food grade compounds since they have to be consumed along with the coatings/films. Additionally, when adding some compounds it is important to determine their impact on coatings/films functionality because there is a possibility of changing their basic functional properties, such as their vapour and gas barrier properties, or solute transport properties. The influence of an ingredient on coatings/films functionality depends on its concentration in the matrix, stability, chemical structure, degree of dispersion in the coating, and also on its interaction with the polymer (Suppakul et al., 2003).

Antioxidants can act in food products by: scavenging free radicals which are responsible for initiating oxidation; inactivating metal ions; removing reactive oxygen species such as oxygen radicals; breaking the initiated chain of reactions; quenching/scavenging singlet oxygen;

destroying peroxides thus preventing radical formation; and removing oxygen and/or decreasing local oxygen concentration/pressure (Eskin & Przybylski, 2001). Oxidation can seriously limit food preservation, its occurrence being negative to both nutritional and organoleptic properties; such deleterious effects can be the change of the nutritional value of food products and also the production of rancidity, colour changes and flavour loss (Nerín et al., 2008). The use of antioxidants can thus be important in food applications, and the coatings/films may provide a very good vehicle for their application.

Synthetic antioxidants have long been used in a variety of foods, but their use has come into dispute due to suspected carcinogenic potential and the general negative response to synthetic food additives by consumers. As a result, there is a growing interest in the characterization of natural antioxidants. Considerable research has been carried out on the assessment of antioxidant properties of many plant extracts when added to coatings and films (Dimitrios, 2006).

2.5 EDIBLE COATINGS/FILMS APPLICATIONS

A large number of polysaccharide biomaterials were used to coat foodstuffs. The application of edible coatings/films to food products can be done by dipping, spraying, brushing or by creating stand alone films from a solution that may subsequently be used to cover food surfaces (Cutter et al., 2002; Pavlath et al., 2009).

Starch is one of the most commonly used agricultural raw materials in biodegradable coatings and films. Starch-based coatings containing potassium sorbate were used to extend the storage life of strawberries. The application of the coating in strawberries reduced the microbial count and extended the storage life from 14 to 28 days (Garcia et al., 1998) and reduced the decay of the fruits when compared with the control (Mali & Grossmann, 2003).

Cellulose and cellulose derivates can form strong and flexible water-soluble films and have been utilized for food packaging. Carboxymethylcellulose was been used to improve storage life of cut apples and potatoes (Baldwin et al., 1996).

It was also found that hydroxypropyl methylcellulose (HPMC) coating with ethanol was effective in inactivating *Salmonella montevideo* on the surface of tomatoes. Application of HPMC coating also retarded the rate of loss of firmness and change in colour of tomatoes stored at 20 °C for up to 18 days (Zhuang et al., 1996).

Recently, HPMC:beeswax coatings have been used to reduced weight and firmness loss of 'Ortanique' mandarins, without compromising flavour quality when compared to uncoated mandarins (Navarro-Tarazaga et al., 2008).

Due to its functional properties chitosan application in edible coatings/films is very attractive. Chitosan films were applied to inhibit the surface spoilage by bacteria in processed meat, using its antimicrobial capacity (Ouattara et al., 2000).

The application of chitosan coating in sliced mango fruit has resulted in retarded water loss, increased soluble solids content and ascorbic acid content (Chien et al., 2007). When applied on whole mango fruits, chitosan coatings extended their shelf-life up to 18 days without any microbial growth and off-flavour (Srinivasa et al., 2002).

Chitosan was used as coating for cucumber and bell pepper fruits, showing that it can be used to reduce water loss and to maintain the quality of the fruits (El Ghaouth et al., 1991).

Polysaccharides extracted from seaweeds were also studied as coatings for food products. Alginate was used to extend the shelf-life of mushrooms (Nussinovitch & Kampf, 1993) and also shows beneficial properties when applied as an edible coating to precooked pork patties (Wanstedt et al., 1981).

Recently, the use of alginate coating on fresh-cut papaya pieces was studied. This coating exhibited slightly improved water barrier properties when compared to the uncoated samples of fresh-cut papaya, and the addition of ascorbic acid as antioxidant in the coatings aided to preserve the natural ascorbic acid content of the fresh cut papaya (Tapia et al., 2008).

Alginate coatings were used to increase the water vapour mass transfer resistance of fresh-cut melon 'Piel de Sapo', preventing dehydration, being calcium chloride used as a cross-linking agent in order to help maintaining fruit firmness (Oms-Oliu et al., 2008).

Carragennan-based coatings were applied to extend the shelf life of strawberry fruits, and is has been shown that the industrial application of calcium-enriched carragennan coatings on fresh strawberries resulted in a decrease in firmness loss when compared to non coated fruits (Ribeiro et al., 2007).

The quality of coated trout fillets after multiple edible coatings application was also studied. Fillets were coated and stored at -18 °C for a period lasting up to 7 months. During the three steps of coating application, gluten in the first-coating group, xanthan gum in the secondary-coating group, and a mixture of 2:1 of wheat and corn flours in the last-coating group were the materials that had the best results. The coated fillets showed low levels of proteolysis and lipid oxidation, and the sensorial properties of the coated fillets were more desirable than those of the noncoated fillets (Kilincceker et al., 2009).

The utilization of galactomannans in lipids formulations has been tested in the last years, in order to improve the lipid films' properties. The incorporation of LBG and GG galactomannans in traditional wax formulations when applied on citrus fruits showed similar results in terms of weight loss. However, the LBG-wax coating produced the best juice, with the best taste quality (Chen & Nussinovitch, 2001).

The shelf-life quality of freshly harvested apples (Golden Delicious) coated with three individually developed lipid/hydrocolloid coatings where LBG was one of the constituents was assessed throughout refrigerated storage. The coatings resulted in low internal O_2 and the least loss of fruit firmness when compared with the non-coated apples group. Sensory analyses showed that the coated apples maintained consistent quality in firmness, crispness and juiciness throughout the storage period (Conforti & Totty, 2007).

Recently 'Fortune' mandarins were coated with LBG-based coatings to extend shelf-life, improve the external appearance and avoid flavour degradation of the fruit. Two experiments were performed: in the first, three LBG–lipid edible coatings were tested and compared with a

commercial wax and the uncoated control. Among the experimental coatings, the coating with beeswax and glycerol and without carnauba wax and olein was the best for controlling weight loss and an improved gloss. The second experiment was designed to optimize the performance of the best coating of the first experiment with two modifications: decreasing emulsion solids content and increasing plasticizer content. Both coating modifications decreased ethanol levels in the juice compared to those from the unmodified coatings. The coating modified by increasing glycerol content showed the best performance in controlling weight loss, improving gloss and reducing ethanol content (Rojas-Argudo et al., 2009).

Table 2-1 presents some of the applications of polysaccharide coatings and films in food products.

2.5.1 Application of coatings and films on cheese

Cheese is a complex food product consisting mainly of casein, fat and water. In cheeses the one of the factors that most affects cheese stability is water activity, which depends mainly on moisture and salt contents. During cheese storage biological reactions due to microorganisms and enzyme activity occur, being the cheese quality influenced by the oxygen and temperature of storage, and gas exchanges with the environment. Cheese releases CO_2 and simultaneously consumes O_2 during its life cycle being required the control of the gas exchange to maintain the cheese quality and increase its shelf-life (Robertson, 2006; Pantaleão et al., 2007).

Also the effects of light and O_2 should be considered in cheese packaging (Robertson, 2006). Several researchers have recommended that fresh cheeses (e.g. cream cheese, decorated cream cheese, soft cheese, and cottage cheese) are packaged in modified atmospheres with N_2 and/or CO_2 replacing the O_2 in the package (Mannheim & Soffer, 1996). However, spoilage caused by yeast and especially bacteria may still occur even at very low O_2 and elevated CO_2 levels (Westall & Filtenborg, 1998). Semi-soft and hard cheeses (whole, sliced or shredded) have a relatively high respiration rate, which require a packaging material somewhat permeable to CO_2 to avoid blowing of the packaging. Meanwhile, O_2 must be kept out to avoid fungal spoilage and oxidation

of the cheese. Instead, these products require a balanced oxygen and carbon dioxide atmosphere to prolong their shelf-life (Haasum & Nielsen, 1998).

Additional environmental factors must be considered in selecting a material for cheese coating (e.g. the light). All these factors affect not only cheese's physical characteristics but also its flavour during storage. In fact, many different compounds contribute to cheese flavour and most of them form during cheese ripening (Robertson, 2006). The cheese protection by coating with synthetic films for moisture regulation and protection against contamination is a well-know procedure being used synthetic materials (cellophane, cellophane-polyethylene, saran, parakote, pliofilm, etc) as coatings (Kampf et al., 2000).

Edible films based on k-carrageenan, alginate and gellan were used to coat a semi-hard cheese and a reduction of weight loss of the coated cheeses was reported. Results showed that cheese coating based on hydrocollloid films improved textural and sensorial properties in comparison with noncoated cheeses. The coating did not influence the taste of white brined cheese, and the coating technology was simple and relatively inexpensive (Kampf et al., 2000). Also, Duan et al. (2007) showed that chitosan-lysozime films and coatings could be applied in Mozzarella cheese packaging to control the post processing microbial contamination, improving the microbial safety of cheese products.

Natural gels (agarose and gellan) were used as coatings to extend the shelf-life of Mozzarella cheese without adding any chemical substances and without thermal procedures (Laurienzo et al., 2006). Recently, an active coating and modified-atmosphere packaging was used to prolong the shelf-life of Fior di Latte cheese. The active coating was based in sodium alginate with lysozyme. The results have shown that combining the active coating with modified-atmosphere packaging resulted in a three days increase of the shelf-life of the cheese (Conte et al., 2009).

Table 2-1. Applications of polysaccharide coatings and films in food

Class	Food	Type	Material	References
Bakery	Crackers	Coating	Corn starch, methylcellulose and soybean oil	(Bravin et al., 2006)
Dairy	Cheese	Coating	k-carrageenan, alginate and gellan	(Kampf et al., 2000)
		Coating and Film	Chitosan	(Duan et al., 2007)
		Coating/ MAP	Alginate	(Conte et al., 2009)
Fruit	Apple	Coating	Alginate and gellan	(Rojas-Graü et al., 2007)
	Mango	Film	Chitosan	(Srinivasa et al., 2002)
	Pear	Coating	Gellan, pectin and alginate	(Oms-Oliu et al., 2008)
	Papaya	Coating	Alginate or gellan	(Tapia et al., 2008)
	Strawberry	Coating	Carragennan	(Ribeiro et al., 2007)
Meat	Ham	Film	Cellulose	(Santiago-Silva et al., 2009)
	Pork	Coating	Pectin	(Kang, Jo et al., 2007)
	Roast beef	Coating	Chitosan	(Beverlya et al., 2008)
Fish	Fish fillet	Coating	Gluten: xanthan gum:wheat and corn flours	(Kilincceker et al., 2009)
	Salmon	Film	Chitosan	(Ye et al., 2008)
Vegetables	Carrot	Coating	Hydroxypropyl methylcellulose	(Villalobos-Carvajal et al., 2009)
	Garlic	Coating	Agar-agar	(Geraldine et al., 2008)
	Potato	Coating	Calcium alginate	(Mitrakas et al., 2008)
	Tomato	Coating	Chitosan	(Casariego et al., 2008)

2.6 CONCLUSION

The utilization of biopolymers as a matrix for the development of edible coatings/films have increased in the last 10 years, however only few works used new sources for the production of the coatings/films. Reported studies showed that a great number of factors may influence the properties of edible coatings/films; and diverse behaviours are observed with the incorporation of plasticizers and/or oil, presenting a great variation depending in the biopolymer used. Therefore, the characterization of edible coatings/films based in new sources or different formulations is necessary, in order to understand how new compounds can be influenced or influence properties of edible coatings/films.

Edible coatings/films have been applied in a great variety of food products, mainly fruits and vegetables; however few studies are available on their application on dairy products.

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CHAPTER 3

EXTRACTION, PURIFICATION AND CHARACTERIZATION OF GALACTOMANNANS FROM NON-TRADITIONAL SOURCES

This chapter presents a methodology for the extraction of galactomannans from seeds of three different species of *Leguminosae* (*Gleditsia triacanthos*, *Caesalpinia pulcherrima* and *Adenanthera pavonina*). The yield of extraction in different stages of the process, monosaccharide composition, as well as physical, chemical and thermal parameters of the isolated galactomannans were determined and compared with previously published results. The results confirm the suitability of the extraction and purification procedure to obtain galactomannans from non-traditional sources.

3.1 INTRODUCTION

In the last decade, there has been a growing interest in the development of thermoplastic materials from biodegradable polymers, particularly those derived from renewable resources. Biobased packaging is defined as packaging containing raw materials originating from biological sources, i.e. produced from renewable, biological raw materials such as starch and bioderived monomers. To date, biodegradable packaging has attracted great attention, and numerous projects are under way in this field. One important reason for this attention is the marketing of environmentally friendly packaging materials. Furthermore, the use of biodegradable packaging materials has the greatest potential in countries where landfill is the main waste management tool (Petersen et al., 1999; Paes et al., 2008).

Galactomannans are present in the endosperm of numerous plants, particularly the *Leguminosae*, and they have several functions, including reserve of carbohydrates (Reid & Edwards, 1995). Galactomannans are polysaccharides built up of a β-(1–4)-D-mannan backbone with single D-galactose branches linked α-(1–6). Their mannose/galactose (*M/G*) ratios differ according to the species (Kök et al., 1999). They are water-soluble hydrocolloids forming highly viscous, stable aqueous solutions (Neukom, 1989). Galactomannans can often be used in different forms for human consumption. Featuring different physicochemical properties, galactomannans are a versatile material used for many applications: they are excellent stiffeners and stabilizers of emulsions, and the absence of toxicity allows their use in the textile, pharmaceutical, biomedical, cosmetics and food industries (Srivastava & Kapoor, 2005; Vieira et al., 2007). Most galactomannans used in pharmaceutical technology and cosmetics are usually unpurified gums (Üner & Altinkurt, 2004). In some occasions galactomannans have been used in binary mixtures with other polysaccharides such as: xanthan gum, agar and k-carrageenan, to form gels with new properties (Fernandes et al., 1991; Bresolin et al., 1999, Vendruscolo et al., 2005).

The three major galactomannans of commercial importance in food and non-food industries are guar gum (GG, *Cyamopsis tetragonolobo*, *M/G* ratio: 2:1), tara gum (TG, *Caesalpinia spinosa*,

M/G ratio: 3:1) and locust bean gum (LBG, *Ceratonia siliqua*, *M/G* ratio: 3.5:1 (Dakia et al., 2008).

Currently the international trends demand the introduction of alternative sources of seed gums (Joshi & Kapoor, 2003) and it is therefore important to search for alternative renewable sources for e.g. the production of edible and biodegradable films and coating materials. In particular, Latin American sources of galactomannans are not well known, in spite of the rich biodiversity of the local flora and of the favourable climate for their production (Azero & Andrade, 2002).

In the present work, three non-traditional galactomannans were isolated from seeds of *Gleditsia triacanthos* (GT), *Caesalpinia pulcherrima* (CP) and *Adenanthera pavonina* (AP) and a simple methodology using only ethanol and water as solvents was developed for their extraction in view of their use e.g. in the demanding area of food industry. All the galactomannans from those plants were obtained by aqueous extraction followed by precipitation with ethanol. Other works used solvents as chloroform (Mirzaeva et al., 1998) and petroleum ether (Üner & Altınkurt, 2004; Amin et al., 2007) to extract galactomannans, which are not authorized in the food industry (List of Codex Specifications For Food Additives).

G. triacanthos belongs to the family *Leguminosae*, grows in America, Middle Europe and Mediterranean area (Üner & Altinkurt, 2004). Galactomannans are the main polysaccharide constituents from the endosperm of the seed of *G. triacanthos* (Manzi et al., 1984).

C. pulcherrima is an ornamental plant found throughout India, but it can be found in other countries as well, especially in Brazil. It also belongs to the family *Leguminosae*. Some of the constituents extracted from *C. pulcherrima* were found to possess anti-tumour (Che et al., 1986; Patil et al., 1997) and antimicrobial properties (Ragasa et al., 2003).

A. pavonina is a plant from the family *Leguminosae*, native from tropical Asia. It is used in reforestation, as ornamental plant and constitutes an important source of wood. The baking of the seeds and of the wood allows its use in the treatment of pulmonary infections, and also in the treatment of chronic ophthalmia (Fonseca & Perez, 2003).

The extraction yield of each of the galactomannans was determined, as well as their monosaccharide composition, *M/G* ratio, purity, intrinsic viscosity and viscosity average molecular mass. They were characterized by methylation analysis and by enzymatic hydrolysis followed by analysis using tandem mass spectrometry (MS/MS) with electrospray ionization (ESI). Their thermal characterization was performed by thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) together with a characterization by Fourier transform infrared (FTIR) spectroscopy.

3.2 MATERIAL AND METHODS

3.2.1. Plant material

The pods of *G. triacanthos* were collected in the Botanic Garden in Porto, Portugal, during January 2006. The pods of *A. pavonina* and *C. pulcherima* were collected in Fortaleza, Federal University of Ceará (Ceará, Brazil) during April 2006. The seeds were manually separated and kept in a cool, dry place until further use.

3.2.2 Polysaccharide extraction

The polysaccharide extraction of *G. triacanthos, C. pulcherrima* and *A. pavonina* was performed with ethanol and distilled water. In this process, the seeds were removed from the pods, cleaned and placed in a blender, where they were mechanically broken. Following this operation, the endosperm was manually separated from the germ and the hull, suspended in ethanol (purity 99.8 %, Riedel-de Haën, Germany) in a proportion 1:3 (seeds:ethanol) at 70 °C during 15 min to inactivate the enzymes and eliminate low-molecular-weight compounds (Egorov et al., 2003; Egorov et al., 2004). The ethanol was decanted and distilled water was added in a proportion of 1:5 (endosperm:water), the suspension was left to rest for approximately 24 h. Then water, in a proportion of 1:10, (suspension:water) was added and mixed in a blender for 5 min.

3.2.3 Polysaccharide purification

The endosperm mixed in the blender was filtered through a nylon net followed by a centrifugation step at 3 800 *g* (Sigma 4K, B. Braun, Germany) during 20 min at 20 °C. The precipitation of the galactomannan was achieved by adding the supernatant to ethanol (purity 99.8 %, Riedel-de Haën, Germany) at a ratio of 1:2. The ethanol was decanted and the precipitated galactomannan was lyophilized and kept in a dry place until further use.

Figure 3-1 shows the flow chart representative of the extraction and purification processes for the galactomannans of the three seeds considered in this work.

3.2.4 Determination of polysaccharide yield

The yield is one of the most economically important aspects of polysaccharide extraction and purification, and it was determined in three stages of the process (*Y1*, *Y2* and *Y3*), for an initial mass of 50 g seeds of each species. *Y1* was calculated dividing the mass of recovered dry endosperm (m_r) by the initial mass of seeds (m_i) thus determine the yield in the stage where the hull and the germ are removed manually, it represents the yield of the pre-treatment process. *Y2* was calculated dividing the result of the difference between the mass of recovered endosperm (m_r) and the mass obtained from the filtration and centrifugation (after drying in a oven until constant weight at 105 °C) (m_f) by the mass of recovered endosperm (m_r), it represents the yield of the purification process. *Y3* represents the total yield of the extraction and purification processes and was calculated dividing the mass of lyophilized galactomannan (m_f) by the initial mass of the seeds (m_i).

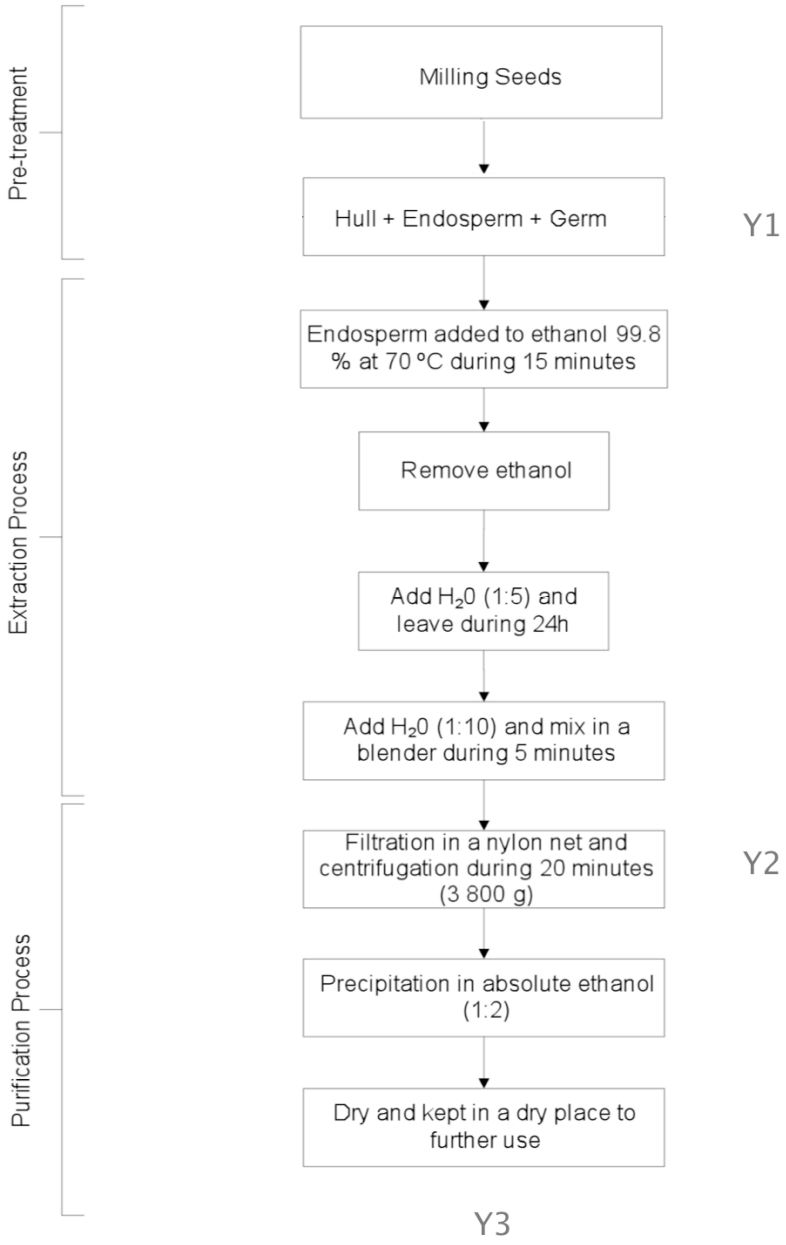


Figure 3-1. Flow chart representative of the extraction and purification processes of the galactomannans. *Y1*, *Y2* and *Y3* are the points of the procedure where the yield was calculated.

3.2.5 Polysaccharide analyses

Polysaccharide analyses were performed as described in Ferreira et al. (2006). Neutral sugars (2 mg) were released through an acid treatment using 0.2 mL 11 M H_2SO_4 for 3 h at 20 °C followed by 2.5 h in 1 M H_2SO_4 at 100 °C, reduced with sodium borohydride, acetylated with acetic anhydride using methylimidazole as catalyst, and the alditol acetates formed were analysed by gas chromatography (Carlo Erba 6000, Carlo Erba, Italy) with a split injector (split ratio 1:60) and a flame ionization detector. The column was a DB-225 (J & W, USA) with 30 m × 0.25 mm and film thickness of 0.25 μm; the oven temperature program was: 220 °C during 5 min, being then the temperature raised at a rate of 20 °C min^{-1} to 230 °C and maintained at this temperature for further 6 min. The flow rate of the carrier gas (H_2) was set at 1 mL min^{-1} at 220 °C. The injector temperature was 220 °C and the flame ionization detector temperature was 230 °C. The hydrolysis of all samples was performed in duplicate and each one was injected twice. Uronic acids were determined by the 3-phenylphenol colorimetric method as described in Ferreira et al. (2006). Samples were prepared in duplicate by hydrolysis in 0.2 mL 11 M H_2SO_4 for 3 h at 20 °C followed by 1 h in 1 M H_2SO_4 at 100 °C. The uronic acids determined were quantitatively accounted as galacturonic acid. The purity of the polysaccharides was evaluated both by the total amount of monosaccharides obtained in the monosaccharide composition and by the amount of manose + galactose present per mg of sample.

3.2.6 Macromolecular characterization

Viscosities of dilute solutions were measured at 25 ± 0.1 °C with a Cannon Fenske capillary viscometer (ASTM-D2515, Series 100), using exactly 10 mL of solution sample. Solutions were prepared to have relative viscosities, η_{rel}, from about 1.2 to 2.0, to assure good accuracy and linearity of extrapolation to zero concentration. The intrinsic viscosity, $[\eta]$, was determined from Huggins' (Eq. 3-1) and Kramer's (Eq. 3-2) equations, where k_H and k_K are the Huggins' and Kramer's coefficients, respectively, η_{sp} is the specific viscosity and C is the solution concentration.

$$\frac{\eta_{sp}}{C} = [\eta] + k'[\eta]^2 C \qquad \text{Eq. 3-1}$$

$$\frac{\ln \eta_{rel}}{C} = [\eta] + k'[\eta]^2 C \qquad \text{Eq. 3-2}$$

Viscosity average molecular masses, *Mv*, were calculated using the Mark–Houwink relationship given by Doublier & Launay (1981) for guar gum as modified by Gaisford et al. (1986) to take into account the different values of *M/G* of the galactomannans.

$$\eta = 11.55 \times 10^{-6} \left[(1-\alpha) \times \overline{Mv}\right]^{1.98} \qquad \text{Eq. 3-3}$$

where $\alpha = 1/ [(M/G) +1]$ and $[\eta]$ is expressed in dL g^{-1}.

3.2.7 Methylation and GC/MS analyses

Polysaccharides were activated with powdered NaOH and methylated with CH_3I (Ciucanu & Kerek, 1984; Isogai et al., 1985) as described by Coimbra et al. (1996). The sample (2–3 mg) was dispersed in 2 mL of dried DMSO and sonicated occasionally until it was fully dispersed. NaOH pellets (100 mg) powdered under argon were added to the solution. The sample was sonicated for 90 min and allowed to stand for an additional 90 min. The methylated material was dissolved in 3 mL of water and extracted with 4 mL of chloroform. The organic layer was washed three times with 3 mL of water, evaporated to dryness, and remethylated to achieve a complete methylation of all free OH groups. The fully methylated material was then hydrolyzed with 2 M trifluoroacetic acid (1 mL) at 121 °C for 1 h, cooled, and rotary evaporated at 35 °C. The partially methylated sugars were then dissolved in 0.3 mL of 2 M NH_3 and 20 mg of $NaBD_4$ were

added. The mixture was allowed to react at 30 °C for 1 h, and the excess of the reducing agent was destroyed by the addition of 0.1 mL of glacial acetic acid. The acetylation of the partially methylated alditols was performed by adding 1-methylimidazole (0.45 mL) and acetic anhydride (3 mL) and allowing to react for 30 min at 30 °C. This solution was treated with water (3 mL) to decompose the excess of acetic anhydride, and the partially methylated alditol acetates (PMAA) were extracted with dichloromethane (3–5 mL). The dichloromethane phase was washed with water and evaporated to dryness. The PMAA were dissolved in dichloromethane (70 μL) and analyzed by GC–MS. GC–MS analysis was performed in a HP series 2 gas chromatograph and Trio-1S VG mass-lab using a DB-1 capillary column (30 m length, 0.32 mm i.d., and 0.25 μm of film thickness). The samples were injected in splitless mode (time of splitless 0.75 min), with the injector and detector operating at 210 and 220 °C, respectively, using the following temperature program: 55 °C for 0.75 min followed by a linear increase of 45 °C min^{-1} until 140 °C, and standing 1 min at this temperature, followed by a linear increase of 2.5 °C min^{-1} until 218 °C, with further 37 min at 218 °C. Linear velocity of the carrier gas (He) was set at 40 cm s^{-1} at 200 °C with a solvent delay of 4 min. MS scans were performed for GC-MS between 400 and 35 *m/z* at 70 eV ionization energy.

3.2.8 Enzymatic hydrolysis

Samples (14 mg) were hydrolyzed with pure endo-β-(1-4)-mannanase (1 U) preparation (Megazyme, EC 3.2.1.78) during 48 h at 37 °C with continuous stirring in a 100 mM Na-acetate buffer pH 5.5 containing 0.02 % sodium azide. The freeze-dried material was dissolved in pyridine-acetate 100 mM buffer pH 5.25, and loaded on a XK 1.6/100 column containing Biogel P-4 (Bio-Rad) previously equilibrated with loading buffer. Fractions (1 mL) were collected and assayed for sugars by static light scattering (Sedere, France). Results were subsequently confirmed by the phenol–H_2SO_4 method (Dubois et al., 1956). The appropriate fractions were pooled and rotary evaporated until all buffer was removed by repeated additions of distilled water, and freeze-dried.

3.2.9 Electrospray ionization mass spectrometry (ESI-MS and ESI-MS/MS)

Freeze-dried fractions obtained after enzymatic hydrolysis and fractionated through Biogel P4 column were dissolved in 200 µL of 1:1 MeOH–water containing 1 % (v/v) formic acid. Samples were introduced into the mass spectrometer using a flow rate of 8 µL min^{-1}. Positive-ion ESI-MS and MS/MS spectra were acquired using a LXQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Typical ESI conditions were: nitrogen sheath gas 30 psi, spray voltage 5 kV, heated capillary temperature 275 °C, capillary voltage 1 V and tube lens voltage 40 V. The precursor ion accumulation time for each scan was controlled by the automatic gain control function of the instrument. ESI-MS/MS experiments were performed on mass-selected precursor ions using standard isolation and excitation procedures (activation *q* value of 0.25, activation time of 30 ms). The collision energy used was between 20-24 (arbitrary units). Data acquisition was carried out with an Xcalibur data system.

3.2.10 Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC)

TGA and DSC measurements were carried out in N_2 atmosphere using a Shimadzu TGA-50 and a Shimadzu DSC-50 equipment (Shimadzu Corporation, Kyoto, Japan) calibrated with Indium as standard, at a heating rate of 10 °C per min. Analyses were started at 20 °C and continued up to 580 °C. Samples were weighed (approximately 5-10 mg of dry matter) in Aluminium DSC pans (Al crimp Pan C.201-52943) being the empty pans used as a reference. Data were treated using TASYS software (Shimadzu Corporation, Kyoto, Japan). Enthalpy was calculated using the area of the peaks between the onset temperature and the end set temperature. For glass transition temperature (T_g) determinations two heating runs were performed between 20 and 250 °C. After the first heating run the samples were left to cool naturally at room temperature under N_2 atmosphere. The second run was used for T_g determination (Mitsuiki, et al., 1998; Dong et al., 2004; Yi & Zhang, 2007).

3.2.11 Fourier transform infrared (FTIR) spectroscopy

IR spectra of the polysaccharides were determined using a Fourier transform infrared spectrometer (FTIR) (Perkin-Elmer 16 PC spectrometer, Boston, USA). The polysaccharide was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into 1 mm pellets for FTIR measurement in the wavenumber range of 400 and 4000 cm^{-1} using 16 scans. Each spectrum was baseline corrected and the absorbance was normalised between 0 and 1.

3.3 RESULTS AND DISCUSSION

3.3.1 Pre-treatment, extraction, purification and global yield

The extraction yield was measured for the processes of polysaccharide pre-treatment (*Y1*), extraction and purification (*Y2*) and for the global process (*Y3*). Table 3-1 shows the results for the yields *Y1*, *Y2* and *Y3*.

Table 3-1. Pre-treatment, extraction and purification and global yields

Species	*Y1* (%)	*Y2* (%)	*Y3* (%)
G. triacanthos	67.13±0.64	42.40±3.51	24.73±2.08
C. pulcherrima	45.27±0.50	66.19±5.89	25.70±3.20
A. pavonina	43.73±1.75	44.67±6.54	17.11±4.15

Y1 is the yield after pre-treatment, where the hull and germ are removed from the endosperm. This yield is a measure of the ease with which hull and germ can be separated from the endosperm. It is also a measure of the relative amount of endosperm in the seed. The highest value of *Y1* (67.13 %) was obtained for *G. triaca*nthos (GT), while *C. pulcherrima* (CP) and *A. pavonina* (AP) had similar values (45.27 % and 43.73 % respectively). These results showed

that the pre-treatment was more effective in removing the hull and germ from the seeds of AP and CP than for the seeds of GT.

The yield *Y2* was measured after the filtration and centrifugation processes; here most of the hull that is still attached to the endosperm after the extraction process is removed. In this stage, CP seeds showed the highest value of yield (66.19 %), while GT presented the lowest value (42.40 %). This is a direct consequence of the previous pre-treatment step: GT endosperm was carrying much more attached material than AP and CP, therefore such material was now removed in more significant amounts, thus decreasing the yield obtained for the first species. The opposite has happened with CP.

The best global yield was obtained for GT and CP, which have values close to 25 % (24.73 % and 25.70 %, respectively). Finally, the variability of the yield results is a direct consequence of the use of different species (Fernandes, 1995). The obtained values for the yield of extraction are in close agreement with those reported in other works (Table 3-2).

Table 3-2. Physicochemical composition of the studied polysaccharides in other works

Species	*M/G*	η (dL g^{-1})	*Mv* x 10^6 (Da)	Yield (%)	References
GT	1.5 – 2.6	-	-	15.4	Manzi et al., 1984
	4.6	-	-	18	Mirzaeva et al., 1998
	1.5 – 3.1	-	-	11.9 – 34.2	Sciarini et al., 2008
	3.2 – 3.5	-	-	15-20	Leschziner & Cerezo, 1970
CP	2.8	13.75	2.1	25	Andrade et al., 1999
AP	1.8	-	-	-	Tavares, 1999

Galactomannans presented a light yellow colour; in fact, even when performing the precipitation in ethanol, some parts of the germ and pigments from the hull pass to the polysaccharide. This observation has been described by other authors, who reported the passage of pigment and

tannins from the hull or from the germ to the endosperm (Avallone et al., 1997; Dakia et al. 2008). The final stage of the global process was the drying step that can determine the colour of the end product. In the present work the polysaccharides were lyophilized, thus minimizing browning and moisture absorption during long-term storage. In other works, as those of Dakia et al. (2008) and Sciarini et al. (2008), the final product was dried in a oven at 100 °C and 35 °C, respectively; the combination of a relatively low water activity and high temperature can enhance the Maillard reaction which provokes browning of the galactomannan, thus changing the galactomannan's chemical properties which in turn may potentially have negative health effects such as those reported to occur in coffee and bread crust, related with acrylamide formation (Frank & Hofmann, 2000).

3.3.2 Polysaccharide composition

Table 3-3 shows the results of polysaccharide analyses which confirmed that mannose (Man) and galactose (Gal) are the major monosaccharides present in the polysaccharide material extracted from GT (66.9 % and 23.7 %, respectively), CP (69.1 % and 24.0 %) and AP (52.8 % and 39.2 %). All the extracted galactomannans contain minor amounts of other monosaccharides such as rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl) and glucose (Glc). There are significant values of Ara monosaccharides (3.0 – 4.5 %). This presence was also shown by Navarro et al. (2002) in the galactomannan of GT and by Nunes et al. (2005) in galactomannans from green and roasted coffee. The presence of these minor components could be attributed to a more complex polysaccharide composition, as single Ara side chains such as those occurring in coffee (Nunes et al., 2005), and/or to contaminants proceeding from the seed coat (Da Silva & Gonçalves, 1990; Dakia et al., 2008).

Table 3-3. Polysaccharide composition of the galactomannans from the studied species

Species	Monosaccharide composition (% mol)							Total Man+Gal	Total (mg/mg)	*M/G*
	Man	Gal	Rha	Fuc	Ara	Xyl	Glc			
GT	66.9±0.9	23.7±0.8	0.0±0.0	1.3±0.6	4.5±0.5	1.4±0.2	1.4±0.2	741.6	808	2.82±0.05
CP	69.1±1.5	24.0±0.1	0.8±0.0	0.8±0.1	3.0±1.4	1.2±0.0	1.2±0.2	841.7	897	2.88±0.07
AP	52.8±0.4	39.2±1.1	1.0±0.3	0.9±0.2	4.2±1.0	1.2±0.2	0.8±0.1	754.7	811	1.35±0.03

The value of *M/G* ratio obtained for GT (*M/G* = 2.82) is in agreement with the values reported in the literature: 3.2 (Leschziner & Cerezo, 1970) and 1.48 – 3.12 (Sciarini et al., 2008). For AP the value of *M/G* reported by Tavares (1999) was high (1.8) when compared with the one obtained in this work (1.35); factors such as the degree of maturation of the seeds, the place of cultivation and differences in the extraction and purification procedures are known to play a determinant role in the *M/G* ratio and may justify the differences found in diverse literature sources. This means that comparisons such as the one made here are useful but should be made with these restrictions in mind. The value of *M/G* obtained for CP (2.9) is close to the value obtained by Andrade et al. (1999), where the extraction process was quite different, with the use of solvents as toluene, acetone and diethyl ether, and with a drying temperature of 35 °C.

In general, galactomannans with higher relative values of Gal monosaccharides are readily soluble in H_2O but have less ability to form gels, while galactomannans with higher relative Man content have the tendency to interact with gelling polysaccharides (Srivastava & Kapoor, 2005).

GT and CP have values of *M/G* very similar to the commercial tara gum (3.0) (Dakia et al., 2008), that is widely used as a thickening agent and stabilizer for food applications.

The results also show (Table 3-3) that the extraction and purification process presented here allows purity values between 80.8 % and 89.7 %, as evaluated from the total monosaccharide content of the sample.

3.3.3 Macromolecular characterization

The determination of intrinsic viscosity provides a measurement of the hydrodynamic volume occupied by the isolated polymer chains in a given solvent and depends primarily on the molecular structure and molecular weight of the polysaccharides as well as on the solvent quality.

The values for k_H depend on solute–solvent interactions and on the state of aggregation of the macromolecules; in a good solvent and for flexible macromolecules, k_H = 0.3; however it can be higher than 1 if aggregation occurs (Sittikijyothin et al., 2005).

Table 3-4 shows the values of physical-chemical composition of the studied galactomannans; k_H values of 1.10 - 1.33 probably reflect some intermolecular aggregation in the samples. In a previous work, Andrade et al. (1999) showed that the galactomannan of CP has an intrinsic viscosity of 13.75 dL g^{-1} and a viscosity average molecular mass of 2.10 x 10^6 Da; however, lower values were found in this work. These differences can be mainly explained by the extraction and purification processes, which are known to influence the intrinsic viscosity and, therefore, the viscosity average molecular mass.

Table 3-4. Physical-chemical parameters of galactomannans extracted from the species considered in the present work (*Mv* – viscosity average molecular mass)

Species	Intrinsic viscosity Huggins´s extrapolation (dL g^{-1})	Intrinsic viscosity Kraemer´s extrapolation (dL g^{-1})	Intrinsic viscosity (average values) (dL g^{-1})	Huggins´ coefficient, k_H	*Mv* (x 10^{-6})
G. triacanthos	10.06	10.78	10.42	1.13	1.62
C. pulcherrima	10.91	11.77	11.34	1.27	1.75
A. pavonina	8.85	9.37	9.11	1.10	1.81

The galactomannans of GT and CP have similar *M/G* ratios (2.82 and 2.88, respectively) but exhibit different intrinsic viscosities (10.42 and 11.34, respectively). For a certain *M/G* ratio, the galactomannans can differ in the distribution of galactose units along the mannan backbone. This distribution, though not fully understood yet, is believed to be important for the functional properties of these polysaccharides (Dakia et al., 2008).

3.3.4 Methylation and GC-MS analyses

Table 3-5 shows the glycosidic linkage composition of the three galactomannans. Partially methylated alditol acetates analysed by GC/MS revealed a polysaccharide containing nonreducing terminal units of Man*p* (0.35, 0.30 and 0.13 %) and Gal*p* (17.23, 20.92 and 34.02 %) as well as 4-Man*p* (59.15, 53.18 and 24.73 %), 4,6-Man*p* (18.93, 22.05 and 36.84 %) and 4-Gal*p* (3.45, 2.70 and 2.95 %), for GT, CP and AP, respectively. The results of the methylation analyses confirm the structure of the galactomannans as a 1-4 mannose with galactose side chains attached at the C6 position. The total content of mannosyl and galactosyl residues is in agreement with monosaccharide composition. Results also show the presence of arabinose residues in galactomannan extracts, as reported in other works (Manzi et al., 1990; Navarro et al., 2002).

The degree of polymerisation of mannose residues for the three galactomannans is within the range usually found for polysaccharide structures (above 100) (Izydorczyk, 2005), showing in this case the greater value for AP galactomannan. GT galactomannan had an estimated average degree of polymerisation of 224 mannose residues, with a degree of branching of 0.24, *C. pulcherrima* had a degree of polymerisation of 252 and a degree of branching of 0.30, and *A. pavonina* had a degree of polymerisation of 475 and a degree of branching of 0.60. These values are in line with the values of the viscosity average molecular weight (*Mv*), showing a positive correlation between the degree of polymerisation and *Mv*, although the values obtained by methylation analysis are much lower (40-80 kDa) than those obtained by viscosity measurements (1.6-1.8 MDa). These differences are possibly due to polysaccharide depolymerisation which may have occurred during methylation, as previously suggested by Nunes and Coimbra (2001).

Table 3-5. Glycosidic-linkage analyses of *G. triacanthos*, *C. pulcherrima* and *A. pavonina* galactomannans

Linkage	Relative abundance (%)					
	G. triacanthos		*C. pulcherrima*		*A. pavonina*	
Terminal-Man*p*	0.35±0.17		0.30±0.09		0.13±0.11	
4-Man*p*	59.15±4.57		53.18±7.16		24.73±0.48	
4,6-Man*p*	18.93±4.51		22.05±3.81		36.84±9.48	
Total Man	78.43±4.07	(66.9)*	75.53±2.86	(69.1)*	61.70±9.19	(52.8)*
DPm	224		252		475	
Terminal-Gal*p*	17.23±4.50		20.92±3.62		34.02±7.80	
4-Gal*p*	3.45±1.03		2.70±0.05		2.95±0.28	
Total Gal	20.69±3.81	(23.7)*	26.21±3.68	(24.0)*	36.97±7.52	(39.2)*
Terminal-Ara*f*	0.55±0.15		0.24±0.22		-	
5-Ara*f*	0.34±0.13		0.51±0.35		0.83±0.65	
Total Ara	0.89±0.27	(4.5)*	0.92±0.06	(1.2)*	0.52±0.34	(0.8)*

*Values of monosaccharide composition (Table 3.3).

3.3.5 Enzymatic hydrolyses and ESI-MS analyses

Galactomannan samples were submitted to *endo*-β-mannanase hydrolyses in order to determine their detailed molecular structure, followed by size-exclusion chromatography (SEC) on a Biogel-P4 column, in order to obtain size-homogeneous oligosaccharide fractions (F_1, F_2 and F_3). The results in the chromatograms corresponding to *G. triacanthos* (GT), *C. pulcherrima* (CP) and *A. pavonina* (AP) samples are shown in Figure 3-2a, 3-2b and 3-2c, respectively. According to the known enzymatic mechanism of *Aspergillus niger endo*-β-mannanase, the hydrolysis of β-(1-4)-linked mannan backbone is hindered by the presence of Gal residues, e.g. the extend of hydrolysis is higher for low galactose-substituted mannan backbones (Daas et al., 2000). The elution profile obtained by SEC after *endo*-β-mannanase hydrolyses showed different abundances of F_3, F_2 and F_1 fractions in each sample. The GT sample showed the highest abundance of low molecular weight fractions, when compared with the other samples, while AP presented the lowest abundance of fractions with lower molecular weight. This pattern can be related with the values of the degree of branching for these polysaccharides. AP galactomannan presents the highest value of degree of branching (lowest abundance of fractions with lower molecular weight) while the GT galactomannan presents the lowest value of degree of branching (higher abundance of fractions with lower molecular weight).

The ESI-MS analyses allow studying intact oligosaccharides, even when present in mixtures and with low abundance, without any manipulation/derivatization being required (Nunes et al., 2005). ESI-MS spectra of oligosaccharide F_1 fractions obtained from GT, CP and AP polysaccharides present similar features. Table 3-6 shows the oligosaccharides identified as $[M+Na]^+$ ions in ESI-MS spectra of galactomannans from GT, CP and AP. The major oligosaccharides of F_1 for each of the three studied galactomannans were identified as the sodium adducts $[M+Na]^+$ of Hex_2 and Hex_3 followed by Hex_4 at *m/z* 365, *m/z* 527 and *m/z* 689, respectively. On GT samples the presence of ions at *m/z* 569 and *m/z* 701, attributed to the acetylated tri- and tetra-saccharides Hex_3Ac_1 and $Hex_3Ac_1Pent_1$, respectively, were detected. To confirm the presence of acetyl and pentosyl groups in GT polysaccharide, F_2 fraction was submitted to a second SEC in Biogel-P4, leading to two fractions F_{21} and F_{22} (Figure 3-3). After this purification step the acetylated groups were still detected, with the presence of ions at *m/z* 893 and 1055, attributed to the acetylated penta- and hexa-saccharides. In these F_{21} and F_{22} fractions,

the sodium adducts $[M+Na]^+$ of Hex_5, Hex_6 and Hex_7 were detected at *m/z* 851, *m/z* 1013 and *m/z* 1175, respectively (Table 3-6). The presence of acetyl groups was observed in other works that used ESI-MS and MS/MS for the characterization of galactomannans (Nunes et al., 2005). In order to confirm the proposed oligosaccharide structures observed in the ESI-MS spectra, these ions were further analyzed by ESI-MS/MS.

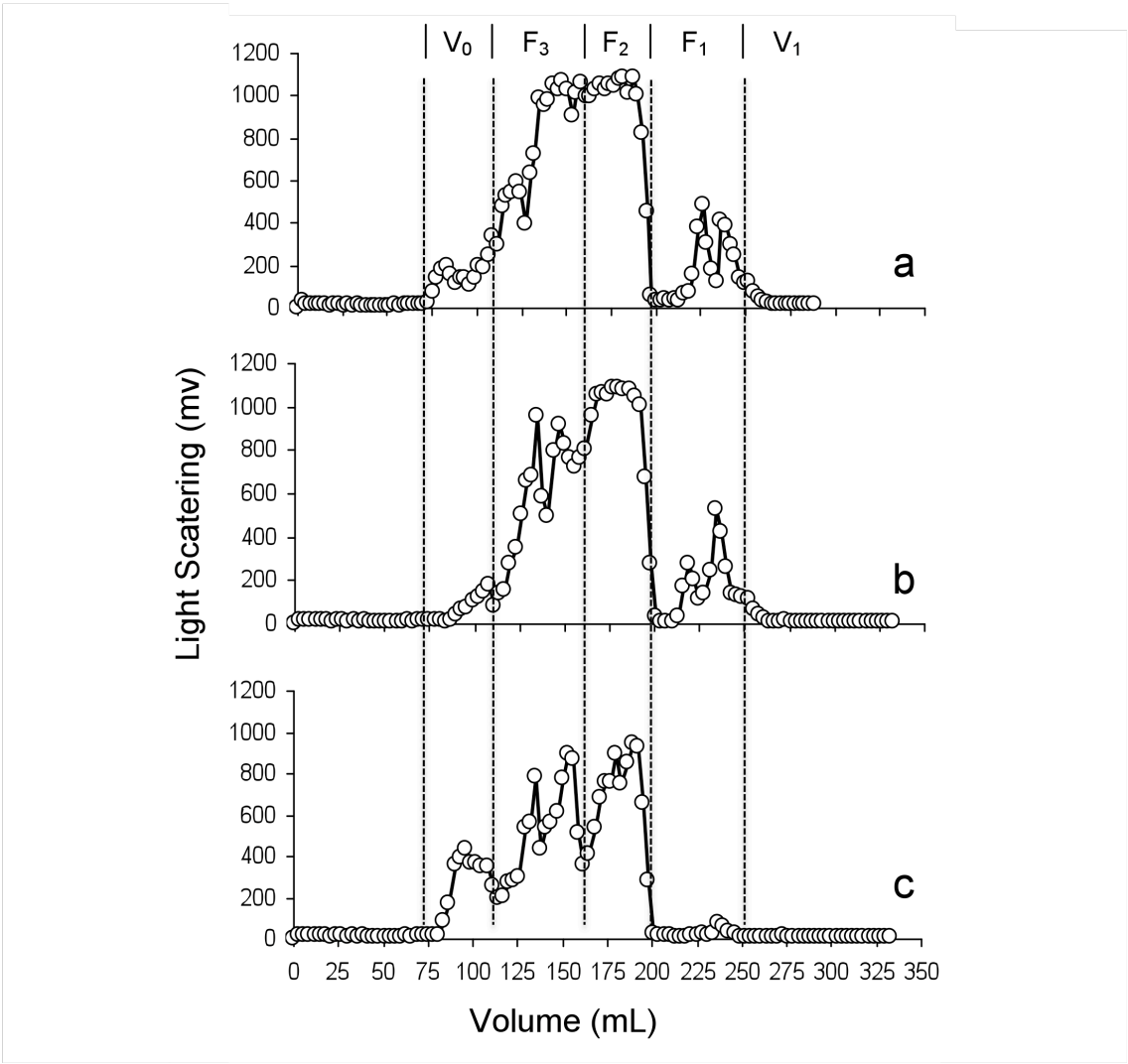


Figure 3-2. Size-exclusion chromatography of oligosaccharides obtained after hydrolysis with *endo*-β-mannanase for (a) *G. triacanthos*, (b) *C. pulcherrima* and (c) *A. pavonina*. V_0 – void volume; V_1 – total volume. F_1, F_2 and F_3 are oligosaccharide fractions with increasing molecular weights.

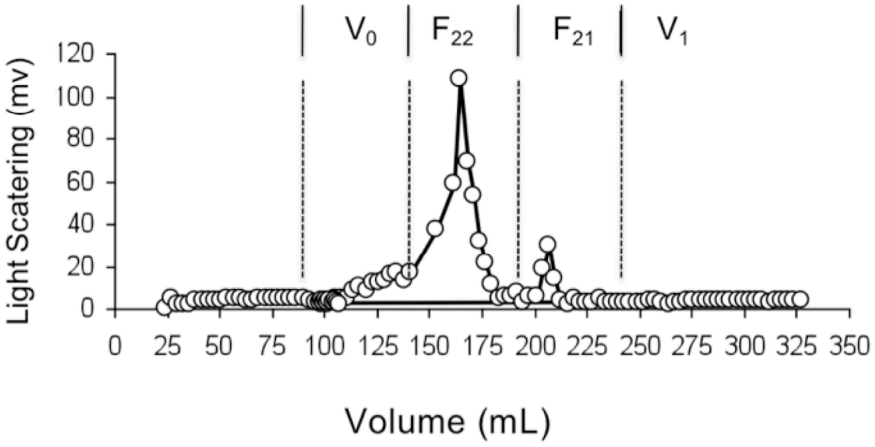


Figure 3-3. Size-exclusion chromatography of fraction F_2 from *G. triacanthos* galactomannan. V_0 – void volume; V_1– total volume. F_{21} and F_{22} are oligosaccharide fractions with increasing molecular weights.

3.3.6 ESI-MS/MS spectra of the obtained oligosaccharides

Oligosaccharide cleavages under ESI-MS/MS conditions are the result of glycosidic cleavages between two sugar residues and of cross-ring cleavages (cleavage of two bonds within the sugar ring).

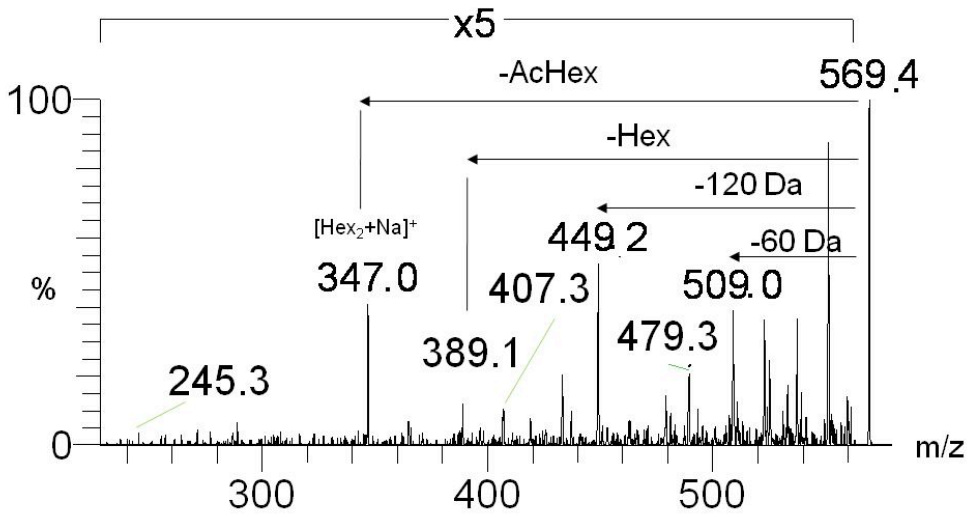
3.3.6.1 *G. triacanthos*

The ESI-MS/MS spectrum of $[Hex_2+ Na]^+$ (*m/z* 365) and $[Hex_3+ Na]^+$ (*m/z* 527) for GT (results not shown) present predominant ions attributed to glycosidic bonds cleavage. The higher intensity of the loss of a neutral fragment with 60 Da is related with the (1-4)-linked hexoses. Also the absence of product ions due to losses of 90 Da, is characteristic of the MS/MS spectrum of 4-linked hexoses (Hofmeister et al., 1991).

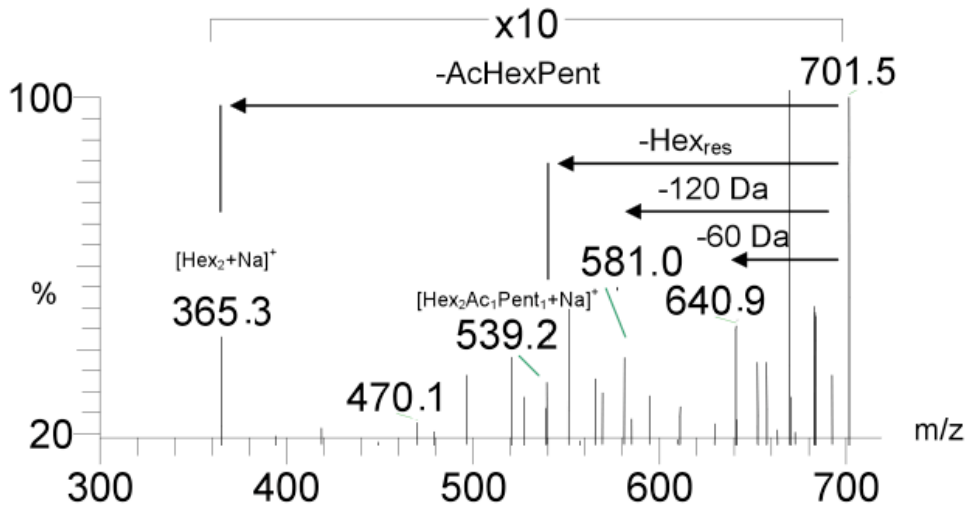
Table 3-6. Oligosaccharide *m/z* ions ([M+Na]+) observed by ESI-MS after hydrolysis with *endo*-β-mannanase of *G. triacanthos*, *C. pulcherrima* and *A. pavonina* galactomannans

Sample	*G. triacanthos*					
n	2	3	4	5	6	7
$[Hex_n+Na]^+$	365	527	689	851	1013	1175
$[AcHex_n+Na]^+$		569		893	1055	
$[Hex_nAcPent+Na]^+$		701				
Sample	*C. pulcherrima*					
n	2	3	4			
$[Hex_n+Na]^+$	365	527	689			
Sample	*A. pavonina*					
n	2	3	4			
$[Hex_n+Na]^+$	365	527	689			

The ESI-MS/MS spectrum of $[Hex_3Ac+ Na]^+$ (*m/z* 569), presented in Figure 3-4a, showed the presence of the ions at *m/z* 389 and *m/z* 347, corresponding to the loss of a Hex unit (-180 Da) and of the loss of one Hex_{res} and plus AcHex (-222 Da), respectively. Although of low intensity, it is observed the ions at *m/z* 407 and *m/z* 245 due the loss of one and two Hex_{res} (162 and 324 Da), respectively. Figure 3-4a also shows the ions at *m/z* 509 (loss of 60 Da), *m/z* 479 (loss of 90 Da) and, with higher abundance, the ion at *m/z* 449 (loss of 120 Da) corresponding to a 1-6 type linkage (Nunes et al. 2005). These fragmentation pathways allowed to identify this oligosaccharide as being composed of two Man units linked by a (1-4) linkage and an additional Gal residue (1-6)-linked, in accordance with methylation analysis. Also, the loss of AcHex (-222 Da) confirms the presence of an acetylated hexose unit.



(a)



(b)

Figure 3-4. ESI-MS/MS spectra of [M+Na$^+$] adducts and schematic fragmentation pathways of Hex_3Ac_1 (a) and $Hex_3Ac_1Pent_1$ (b) for fraction F_1 of *G. triacanthos*.

The ESI-MS/MS spectrum of $[Hex_3AcPent+Na]^+$ (*m/z* 701) (Figure 3-4b), show the product ion at *m/z* 539 corresponding to the loss of one Hex_{res} (-162 Da), and at *m/z* 365, attributed to the loss of one block HexAcPent. To confirm the presence of acetyl groups on this galactomannan structure the oligosaccharides at m/z 893 and 1055 observed in the fraction F_{22} were analysed by ESI-MS/MS (Figure 3-5).

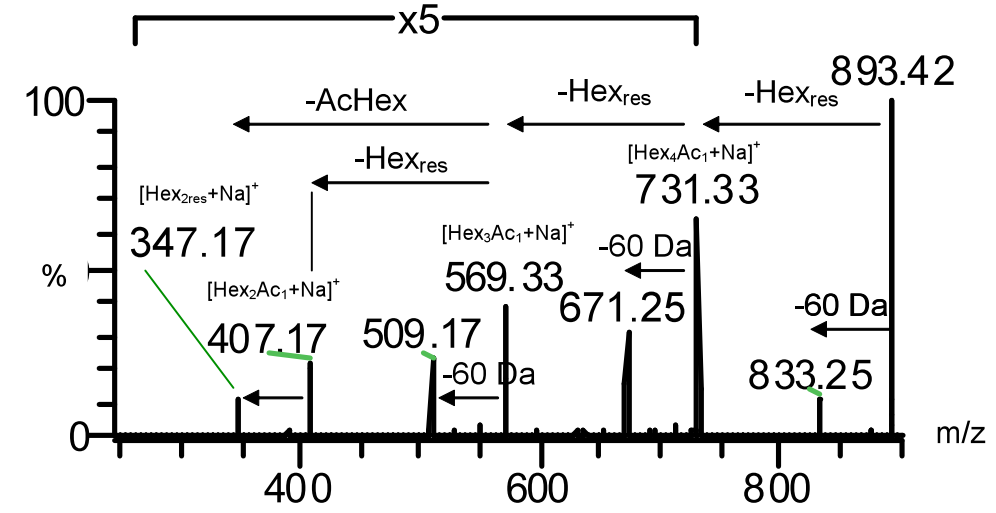
Figure 3-5a shows the ESI-MS/MS spectrum of $[Hex_5Ac+ Na]^+$ (*m/z* 893), that confirms the presence of ions at *m/z* 731, *m/z* 569 and *m/z* 407 due the loss of one, two and three 162 Da (Hex_{res}), respectively, by a *C/Y*-type cleavage. The ion *m/z* 347 was formed by the combined loss of a Hex unit (180 Da) and of one AcHex (222 Da).

The ESI-MS/MS spectrum of $[Hex_6Ac+ Na]^+$ (*m/z* 1055) (Figure 3-5b), shows the presence of ions *m/z* 893, *m/z* 731, *m/z* 569 and *m/z* 407 due the loss of one, two, three and four 162 Da (Hex_{res}), respectively. The ion *m/z* 347 corresponds to the loss of AcHex (222 Da), plus loss of three hexose residues. These results indicate that mannans isolated from galactomannan of GT contain hexose residues bearing one acetyl group.

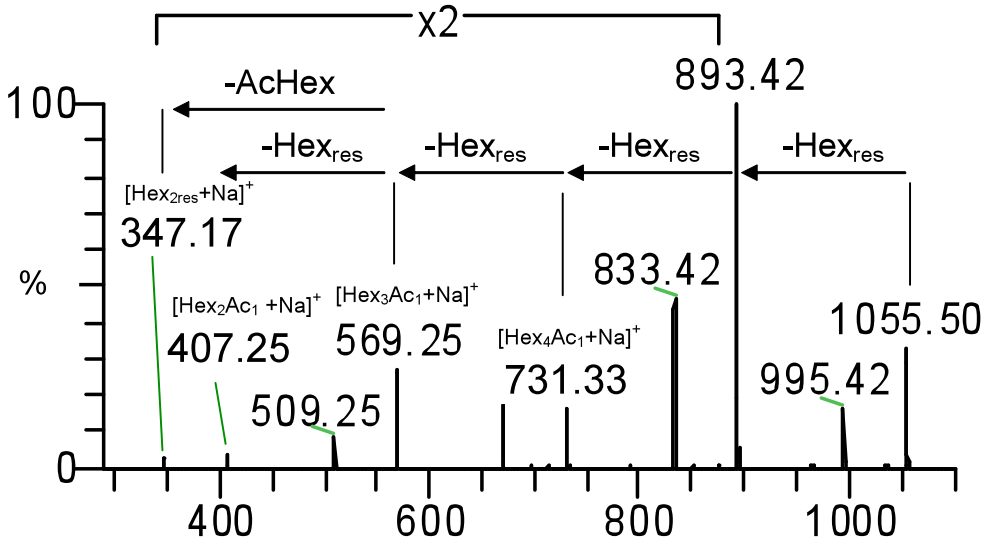
3.3.6.2 *C. pulcherrima* and *A. pavonina*

The ESI-MS/MS spectrum of $[Hex_2 + Na]^+$ (*m/z* 365) and $[Hex_3+ Na]^+$ (*m/z* 527) (data not shown), showed the presence of predominant ions attributed to *C/Y*-type glycosidic bond cleavage. The ESI-MS/MS spectrum of $[Hex_3+ Na]^+$ and $[Hex_4+ Na]^+$ of AP samples (data not shown), corresponds to tri- and tetra-saccharide $[M + Na]^+$ ions, respectively. ESI-MS/MS spectra of the ions at *m/z* 527 and *m/z* 689 show loss of one and two Hex_{res} respectively, confirming that they are oligosaccharides composed by hexose residues. The loss of 60 Da, with higher abundance, in the ESI-MS/MS spectrum of $[Hex_3+Na]^+$ is a characteristic of the MS/MS spectrum of (1-4)-linked hexoses. Also the ESI-MS/MS spectrum of $[Hex_4+ Na]+$, presents losses of 60 Da, characteristic of the MS/MS spectra of (1-4)-linked hexoses.

The analyses of lower molecular weight fractions (F_1) of the three galactomannans show similar structures, although some differences such as the presence of acetyl and pentosyl groups in GT galactomannan can be noticed.



(a)



(b)

Figure 3-5. ESI-MS/MS spectra of [M+Na+] adducts and schematic fragmentation pathways of Hex_5Ac_1 (a) and Hex_6Ac_1 (b) for fraction F_{22} of *G. triacanthos*.

3.3.7 Differential scanning calorimetry (DSC) and Thermogravimetric analyses (TGA)

DSC and TGA analyses were performed in order to understand the thermal behaviour of galactomannan and how can it be influenced by polysaccharide structure. DSC was used for studying thermal transitions occurring in the course of heating under an inert atmosphere. DSC thermograms present typical natural polysaccharides DSC plots (Zohuriaan & Shokrolahi, 2004; Chairez-Matínez et al. 2008; Vendruscolo et al., 2009). Peak temperatures for the various thermal effects as well as the associated enthalpy changes for all the polysaccharides studied are given in Table 3-7. Most of the DSC traces exhibited early endothermic events in the temperature range between 99 and 224 °C, which can be explained by water evaporation. Similar results were observed in other galactomannans: Vendruscolo et al. (2009) reported an endothermic event near 100 °C and Chaires-Martinez et al. (2008) detected peaks between 117.64 and 119.71 °C. The second event was related to an endothermic peak between 306.59 and 345.56 °C (Table 3-7).

Some differences can be observed between samples in the DSC thermograms (Figure 3-6). The enthalpy change for the first thermal transition (ΔH_1) increased by 8.7 % from AP to GT, being the CP sample the polysaccharide with the highest ΔH_1 value. These increases have a direct relationship with the values of intrinsic viscosity and of the mannose:galactose ratio of the samples, in agreement with mannose molar content of GT, CP and AP samples of 66.9, 69.1 and 52.8 % mol, respectively. Na and Lee (1997) showed that the energy required for the thermal transition of lactan gum was related to the steady shear viscosity ranking of gum samples. The increase of mannose content in the sample indicates a lower branching and a higher binding energy between the backbone monosaccharides (Na & Lee, 1997); similar results were reported by Chaires-Martínez et al. (2008) who compared guar gum with a lower content of *M/G* ratio with locust bean gum, having guar gum displayed lower values of ΔH_1.

Table 3-7. Peak temperatures (T_1 and T_2) in DSC thermograms, enthalpy changes (ΔH_1 and ΔH_2) and glass transition temperature (T_g) of galactomannan samples

Sample	Peak nº	Onset (°C)	T_1 (°C)	T_2 (°C)	Endset (°C)	ΔH_1 (J g^{-1})	ΔH_2 (J g^{-1})	T_g* (°C)
G. triacanthos	1	102.05	137.11	-	205.72	324	-	56.4
	2	311.09	-	322.41	337.13		33.98	
C. pulcherrima	1	101.52	139.18	-	203.56	326		66.9
	2	313.65	-	329.83	344.30	-	34.79	
A. pavonina	1	112.80	151.36	-	208.13	298		52.6
	2	306.59	-	328.16	345.56	-	47.75	

*Obtained by the second heating run.

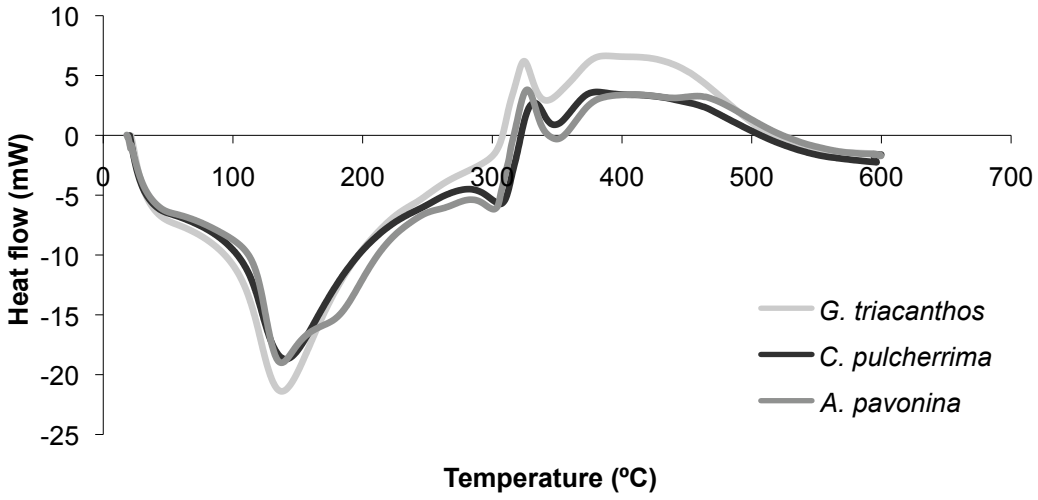


Figure 3-6. DSC scans (1st run) of the three analyzed galactomannans obtained at a heating rate of 10 °C min^{-1} under nitrogen atmosphere.

Table 3-7 shows the values of enthalpy change for the second thermal transition, ΔH_2. The values obtained for galactomannans of GT, CP and AP were 33.98, 34.79 and 47.75 J g^{-1}, respectively. These values are related to the samples' thermal decomposition and are in agreement with the viscosity-based average molecular weight (*Mv*) and with the degrees of polymerisation and branching of the samples (Riande et al., 2000, Sperling, 2006), which are both higher for AP galactomannan.

Glass transition temperature (T_g) is a parameter associated with the system's mobility, and is defined as a physical change from a glassy to a rubbery state in amorphous materials promoted by heat or addition of a plasticizer (Roos & Karel, 1991). T_g values of polymers can be explained by a great number of reasons as molecular weight, crystallinity and intermolecular bonding (Sperling, 2006). Chairez-Martínez et al. (2008) attribute the differences in T_g values of galactomannans to the *M/G* ratio and to the distribution of galactose units in mannose chains. Table 3-7 shows T_g values for the galactomannans under consideration; these values show that a higher *M/G* ratio leads to higher values of T_g. These values are close to those reported in the literature for other galactomannan sources (Yi & Zhang, 2007; Chairez-Martínez et al., 2008).

Also TGA curves (Figure 3-7) are in agreement with other works on galactomannans (Varma et al., 1997; Zohuriaan & Shokrolahi, 2004; Vendruscullo et al., 2009), showing two mass loss events for all polymers. The first occurs near 100 °C and may be attributed to the loss of adsorbed and structural water, which in turn is associated to the hydrophilic nature of the functional groups of each polysaccharide. The second mass loss event resulted in a weight loss of ca. 45 %, due to polysaccharide thermal decomposition, presenting peaks of the derivate of the weight loss curve (*DTG*) at 309.81 °C, 321.73 °C and 320.62 °C for GT, CP and AP, respectively (Table 3-8). These values are in conformity with the values presented by Vendruscullo et al. (2009) for the galactomannan of *Mimosa scabrella*, with values ranging between 299.7 and 311.9 °C, depending on the drying temperature. Also Varma et al. (1997) presented a *DTG* value of 306 °C for guar gum.

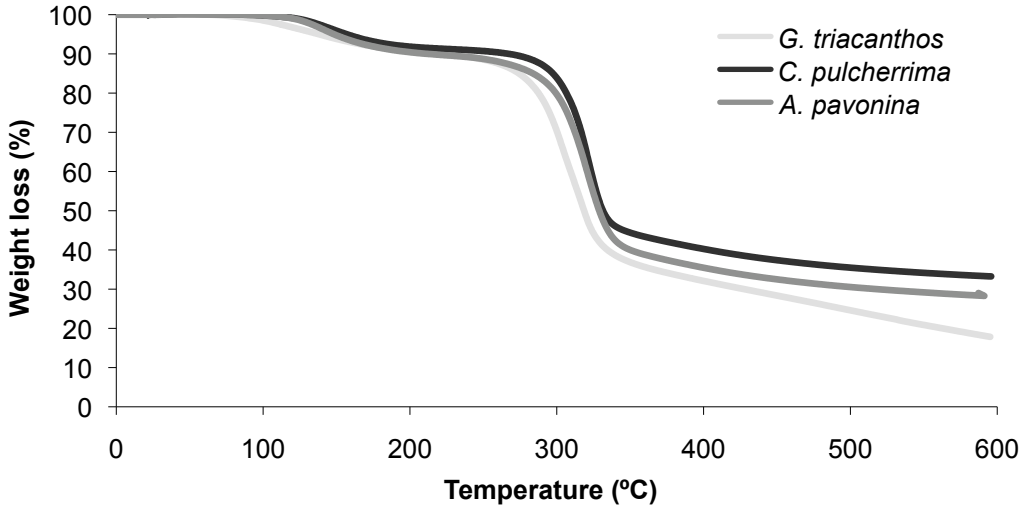


Figure 3-7. Thermogravimetric curves for the three analyzed galactomannans obtained at a heating rate of 10 °C min^{-1} under nitrogen atmosphere.

Table 3-8. Thermogravimetric data for *G. triacanthos*, *C. pulcherrima* and *A. pavonina* galactomannan samples

Sample	Peak	*DTG* (°C)
G. triacanthos	1	136.89
	2	309.81
C. pulcherrima	1	143.32
	2	321.73
A. pavonina	1	141.85
	2	320.62

3.3.8 Fourier transform infrared (FTIR) spectroscopy

Figure 3-8 shows the FTIR spectrum of the analyzed galactomannans. The IR spectra of galactomannans shows peaks at 815 and 872 cm^{-1} that are related with the presence of anomeric configurations (α and β conformers) and glycosidic linkages, attributed to α-D-galactopyranose units and β-D-mannopyranose units, respectively (Figueiró et al., 2004; Prado et al., 2005; Yuen et al., 2009). The broad band between 1198 and 983 cm^{-1} results from the stretching vibration of C-O in C-O-H bonds (e.g. glycosidic bonds) and is related with the galactomannans' sugar composition. The peak at 1152 cm^{-1} corresponds to bending vibrational modes of C–O, present in the pyranose ring, while the broad band between 1134-983 cm^{-1} is a characteristic contribution of C–OH bending (Figueiró et al., 2004; Prashanth et al., 2006). The broad bands ranging between 2800-3000 cm^{-1} and 3100-3500 cm^{-1} are attributed to C-H stretching and to O-H stretching vibration, respectively (Yuen, et al. 2009).

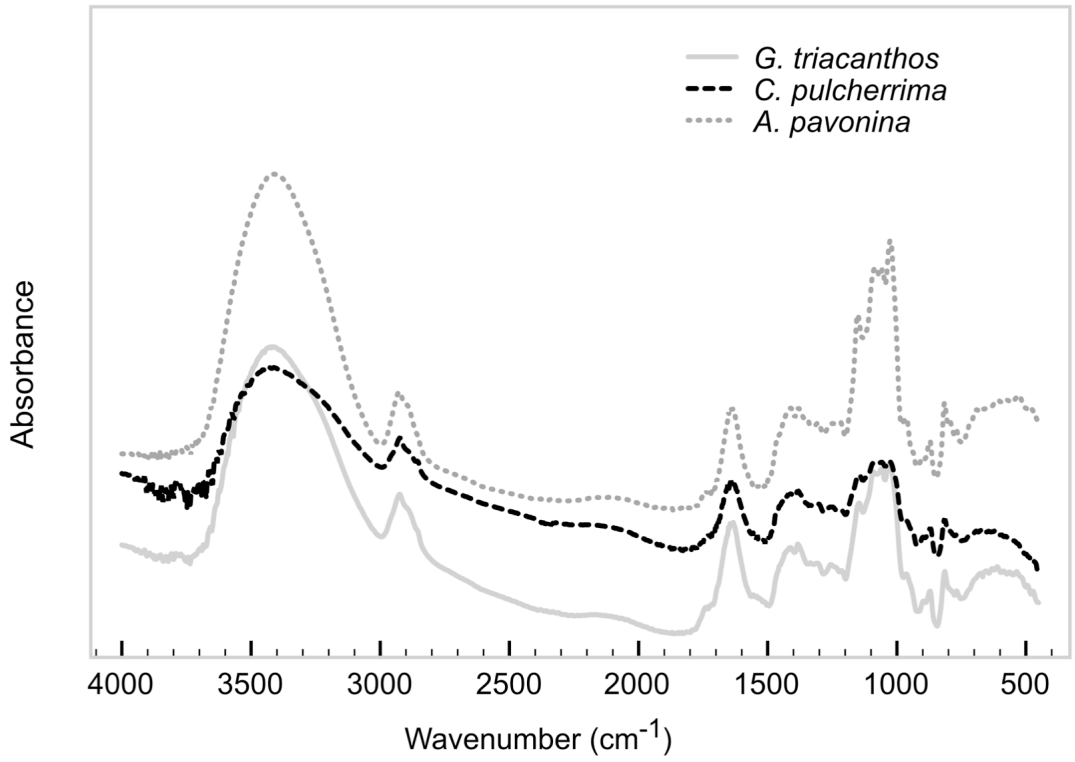


Figure 3-8. FTIR spectra of the three analyzed galactomannans in the spectral region between 400 and 4000 cm^{-1}.

3.4 CONCLUSIONS

Galactomannans are used by the industry under commercial formulations and new sources are important as an alternative to the traditional galactomannan sources from where such formulations are derived. In this work galactomannans were extracted and purified from the seeds of three species of plants from the family *Leguminosae* through an extraction and purification procedure, which uses only ethanol and water. The yields of extraction for the studied galactomannans, starting from the seeds, were: 24.73 %, 25.70 % and 17.11 % for *G. triacanthos*, *C. pulcherrima* and *A. pavonina*, respectively.

These three galactomannans are characterized by a 1-4 linked mannose polymer with galactose side chains attached at the C6 position. Enzymatic hydrolysis with endo-β-mannanase followed by analyses by ESI-MS/MS indicate the presence of acetyl and pentose groups in the galactomannan of *G. triacanthos*. Thermal analyses have shown that the galactomannan compositions influence their thermal behaviour, existing a relation between mannose content, viscosity-based average molecular weight, degree of polymerisation and branching of the samples and their thermal transitions events and glass transition temperature.

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CHAPTER 4

SEED EXTRACTS OF *GLEDITSIA TRIACANTHOS*: FUNCTIONAL PROPERTIES EVALUATION AND INCORPORATION INTO GALACTOMANNAN FILMS

In this chapter, three different extraction procedures were performed in *Gleditsia triacanthos* seeds in order to obtain extracts that were characterized in terms of yield of extraction, total phenolic content and antioxidant properties. Different concentrations of the selected extract (extract presenting simultaneously the best values of total phenolic content, radical scavenging activity and the concentration of the compounds that caused a 50 % inhibition of the radical scavenging activity) were incorporated into *G. triacanthos* galactomannan film forming solutions and films were cast from these and were used to evaluate film properties as: water vapour permeability, colour parameters, total phenolic compounds content and antioxidant activity.

4.1 INTRODUCTION

Nowadays there is an increasing interest in identifying antioxidant properties in products from natural sources to be used e.g. in food preservation or in health-enhancing foods. Edible coatings/films can provide additional protection for food, while being a fully biodegradable, environmental friendly packaging system. They can be used to improve food quality and prolong shelf-life of fresh products and they can be used to carry functional ingredients such as antioxidants, antimicrobials, nutrients, and flavours to further enhance food stability, quality, functionality and safety (Lin & Zhao, 2007). In addition, there is a very strong interest in identifying antioxidant properties from products of natural sources in view of their application in food preservation: e.g., active substances such as plant extracts can be added, conferring e.g. antimicrobial and/or antioxidant properties to the foods (Lee, 2005).

Antioxidants can be incorporated into or coated onto food packaging materials to control the oxidation of fatty components and pigments, and can contribute to the quality preservation of foods; some antioxidants incorporated into plastic packaging materials may have the dual role of protecting the polymer as well as the packaged food from oxidation (Lee, 2005). The number of studies related with residual sources of active compounds has been increasing considerably driven by the high interest of the agro and food industry, and fostered by the increasing amount of information about the specific location of active compounds and their modification during processing (Demo et al., 1998; Jayaprakasha et al., 2003; Xanthopoulou et al., 2009).

Many publications reported that seeds could be considered a valuable source of phenolics, showing to possess beneficial activities such as antioxidant, anti-carcinogenic, antimicrobial, anti-mutagenic and anti-inflamatory (Al-Farsi & Lee, 2008). Recently, numerous reports have described antioxidants and compounds with radical-scavenging activity present in fruits, seeds, vegetables, herbs and cereals extracts (Majhenič et al., 2007; Al-Farsi et al., 2008; Subhasree et al., 2009).

The studies of natural antioxidants with edible coatings/films are increasing in the latest years, and a great number of works report protein-based films as the principal material used but only a few use polysaccharide-based films as the main material (Seydim & Sarikus, 2006; Sivarooban et

al., 2008; Han et al., 2008; Gómez-Estaca et al., 2009; Nuthong et al., 2009; Gómez-Estaca et al., 2009). To our knowledge, no research has been performed on galactomannan active films with antioxidant properties.

Seeds extracts from *Gleditsia triacanthos* have been used in the cosmetic industry by GreenTech S.A. and is commercial available as *Gleditschia*. The hydroglycolic extract of *G. triacanthos*, containing an alkaloid (triancanthine), galactomannans, flavonoids and tannins is used for hair protection and fixing and in capillar care applications (see, e.g., Chemidex, 2008).

Antimicrobial and antioxidant packaging systems containing natural active substances may have high potential for commercial food packaging applications. Consumers clearly prefer an improved food safety of fresh products and minimally processed foods using this type of packaging systems. Most materials containing natural active agents are more effective when there is a direct contact of the packaging materials with the food product. For an effective introduction of new packaging systems into the market, careful design is required. Natural antimicrobials and antioxidants are usually costly, and therefore further development of package design using the minimum of active agents is desirable for practical applications (Lee, 2005).

The work presented in this chapter had three objectives: the first one was to evaluate the total phenolic content and antioxidant properties of ethanolic and water extracts from *G. triacanthos* seeds; the second one was to demonstrate whether galactomannan films are suitable to incorporate antioxidant compounds for further application in the food industry; and thirdly, to show how the main film properties can change with galactomannan and extract concentrations. Galactomannan from *G. triacanthos* has been chosen as main material for edible coatings/films based on their yield of extraction and their availability when compared with the others galactomannan sources.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

G. triacanthos seeds were collected in the Botanic Garden of Porto, Portugal, in November 2007. The Folin–Ciocalteau reagent (Panreac, Spain), Na_2CO_3 (Fluka, Germany) and gallic acid (Sigma, Germany) where used in the quantification of the total phenolic content. Antioxidant determinations were performed using 2,2-diphenyl-1-picrylhydrazyl (Sigma, Germany), 2,6-Di-tert-butyl-4-methylphenol (BHT) (Fluka, Germany), 3-ter-Butyl-4-hydroxyanisole (BHA) (Fluka, Germany) and methanol P.A. (Riedel-de Haën, Germany). The reagents for film formulations were glycerol 87 % (Panreac, Spain) and distilled water.

4.2.2 Extraction from galactomannan and extracts recovery

The polysaccharide extraction was performed as described in Chapter 3 (section 3.2.2) with some changes in the pre-treatment processes, as described below.

4.2.2.1 Pre-treatment A

In this process, the seeds were removed from the pods, cleaned and placed in a blender, where they were mechanically broken into a coarse size (ca. 0.2 to 0.5 mm length). Following this operation, they were suspended in ethanol (purity 99.8 %, Riedel-de Haën, Germany) in a proportion of 1:3 (seeds:ethanol) at 70 °C during 15 min. The ethanol was decanted and the endosperm was manually separated from the germ and the hull. This ethanolic fraction was called extract A.1. Then distilled water was added in a proportion of 1:5 (endosperm:water) and the suspension was left to rest for approximately 24 h. Then water, in a proportion of 1:10, (suspension:water) was added and mixed in a blender for 5 min. In this case no water extraction was performed (as in pre-treatments B and C) and this fraction (A.2) was integrally transferred for the purification process (described in section 4.2.2.4).

4.2.2.2 Pre-treatment B

Also here the seeds were removed from the pods, cleaned and placed in a blender, where they were mechanically milled (until a fine powder was obtained). All the milled seeds were suspended in ethanol (purity 99.8 %, Riedel-de Haën, Germany) in a proportion of 1:3 (seeds:ethanol) at 70 °C during 15 min. The ethanol was decanted (B.1) and distilled water was added in a proportion of 1:5 (endosperm:water), and the suspension was left to rest for approximately 24 h. The water left during 24 h was removed being called B.2. Then water, in a proportion of 1:15 (suspension:water) was added and mixed in a blender for 5 min.

4.2.2.3 Pre-treatment C

Also here the seeds were removed from the pods, cleaned and placed in a blender, where they were mechanically milled (until a fine powder was obtained). All the milled seeds were suspended in ethanol (purity 99.8 %, Riedel-de Haën, Germany) in a proportion of 1:3 (seeds:ethanol) at 70 °C during 6 hours (using a soxhlet system). The ethanol was decanted (C.1 fraction) and distilled water was added in a proportion of 1:5 (endosperm:water), the suspension was left to rest for approximately 24 h. The water left during 24 h was removed being called C.2. Then water, in a proportion of 1:15 (suspension:water) was added and mixed in a blender for 5 min.

4.2.2.4 Galactomannan purification

All the purification processes were performed in the same way. The endosperm mixed in the blender at the end of each pre-treatment performed, was filtered through a nylon net followed by a centrifugation step at 3800 *g* (Sigma 4K, B. Braun, Germany) during 20 min at 20 °C. The precipitation of the galactomannan was achieved by adding the supernatant to ethanol (purity 99.8 %, Riedel-de Haën, Germany) at a ratio of 1:2. The ethanol was decanted and the precipitated galactomannan was lyophilized and kept in a dry place until further use. The ethanol decanted in this process was called extract A.3, B.3 and C.3 depending of the pre-treatment used.

4.2.2.5 Extract recovery

All the extracts from the different extraction steps were filtered (GF/F, Whatman filter paper), and concentrated in a rotary evaporator at 60 °C. The extracts were stored in the dark at 5 °C until further use.

4.2.3 Determination of total phenolic content (TPC)

The determination of *TPC* in extracts and films was done by a UV-Vis spectrophotometer (Varian UV–VIS Spectrophotometer, Germany), based on a colorimetric oxidation/reduction reaction, as described by Skerget, Kotnik, Hadolin, Rizner-Hras, Simonic and Knez (2005). The oxidizing reagent used was Folin–Ciocalteu reagent. To 0.5 ml of diluted extract (20 mg of extract in 10 ml distilled water) or film (20 mg of film in 10 ml distilled water) a volume of 2.5 ml Folin–Ciocalteu reagent (diluted 10 times with water) and 2 ml of Na_2CO_3 (75 g L^{-1}) (Riedel-de Haën, Germany) were added. Absorbance was measured at 760 nm after 30 min incubation at 25 °C and the results were expressed as g of gallic acid from the calibration curve previously carried out with this reagent (Absorbance = 6.5630 × Concentration (g L^{-1}) + 0.0968, R^2=0.9993). All experiments were carried out in triplicate. The total phenolic content was determined as gallic acid equivalents and values are expressed as mg of gallic acid per g of extract.

4.2.4 Antioxidant activity

The DPPH-scavenging potential of the different extracts and films was measured, based on the scavenging ability of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by *G. triacanthos* extracts. The ability of extracts to scavenge DPPH radicals was determined by the method of Blois (1958). Briefly, 1 ml of methanolic solution of DPPH (1mM) (Riedel-de Haën, Germany) was mixed with 1 ml of extract solution (containing 0.5–5.0 mg mL^{-1} of dried extract) or film solution (containing 0.5–5.0 mg mL^{-1} of film). The mixture was then vortexed vigorously and left for 30 min at room temperature in the dark. The absorbance was measured at 517 nm (Varian UV–

VIS Spectrophotometer, Germany) and the activity was expressed as percentage DPPH-scavenging activity relative to the control, using the following Equation:

$$\%\, Radical\ scavenging\ activity\ (RSA) = \left[\frac{(A_{control} - A_{sample})}{A_{control}}\right] \cdot 100 \qquad \text{Eq. 4-1}$$

where $A_{control}$ represents the absorbance value of the control sample and A_{sample} represents the absorbance value of the analyzed sample. The IC_{50} value was also calculated as the concentration of the compounds that caused a 50 % inhibition of the radical scavenging activity (*RSA*). All experiments were carried out in triplicate.

4.2.5 Determination of polysaccharide and extracts yield

The polysaccharide yield (*PY*) was determined at the end of the purification for an initial mass of 50 g seeds. *PY* (Eq. 4.2) represents the total yield of the extraction and purification processes and was calculated dividing the mass of lyophilized galactomannan (m_l) by the initial mass of the seeds (m_i), expressed as g of galactomannan per 100 g of seeds (% w/w). The extracts yield (*EY*) was determined at the end of each recovered process p_i (where *p* is the pre-treatment used A, B and C, and $_i$ the number of the process 1 (for process A), 2 (for process B) and 3 (for process C)). *EY* (Eq. 4.3) was calculated dividing the mass of the recovered extract (m_{re}) by the initial mass of seeds (m_i), being expressed as g of extract per 100 g of seeds (% w/w).

$$PY = \frac{m_l}{m_i} \cdot 100 \qquad \text{Eq. 4.2}$$

$$EY = \frac{m_{re}}{m_i} \cdot 100 \qquad \text{Eq. 4.3}$$

4.2.6 Film preparation

The film formulations were based in a 2^2 level factorial design with a centre point. Galactomannan concentrations of 0.5, 1.0 and 1.5 % (w/v) were used, together with extract concentrations of 0.0, 0.5 and 1.0 % (w/v) (Table 4-1). A concentration of 0.5 % (w/v) of glycerol was used in all films formulations. The extract used in the film formulation was selected based on the results presented in Table 4-2; the choice was made taking into account the extract presenting simultaneously the best values of *TPC*, *RSA* and IC_{50}. The films were prepared by dissolving the lyophilized galactomannan in distilled water (20 °C). Each mixture of galactomannan and glycerol was stirred for 2 h at room temperature (20 °C) and the seeds extracts were added at the corresponding concentration; the obtained suspensions were homogenized with an Ultra-Turrax homogenizer (T 25, Ika-Werke) in two cycles of 1 minute at 5000 rpm. To produce the films, a constant amount (13 mL) of film forming solution was cast onto a 5.7 cm diameter Petri plate. The films were dried in an oven at 35 °C for 16 h and maintained at 20 °C and 0 % RH, at least 24 h before performing the tests.

Table 4-1. Galactomannan and extract concentration used in films formulation, and coded levels associated to factorial design, together with the values of films thickness.

Film	Galactomannan concentration (%) (w/v)	Extract concentration (%) (w/v)	Thickness (mm)
GT1	0.5 (-1)	0.0 (-1)	0.045±0.001[a]
GT2	0.5 (-1)	1.0 (+1)	0.110±0.004[b]
GT3	1.5 (+1)	0.0 (-1)	0.052±0.002[c]
GT4	1.5 (+1)	1.0 (+1)	0.105±0.006[b]
GT5	1.0 (0)	0.5 (0)	0.089±0.012[d]

[a-d] Different superscript letters in the same column indicate a statistically significant difference (Tukey test $p<0.05$).

4.2.7 Film thickness

The film thickness was measured with a digital micrometer (No. 293-5, Mitutoyo, Japan). Five thickness measurements were taken on each testing sample in different, randomly chosen points. The mean values were used to calculate water vapour permeability (*WVP*) and are presented in Table 4-1.

4.2.8 Water vapour permeability (WVP) measurement

The measurement of water vapour permeability (*WVP*) was performed gravimetrically based on ASTM E96-92 method (Guillard et al., 2003). The film was sealed on the top of a permeation cell containing distilled water (100 % RH; 2337 Pa vapour pressure at 20 °C), placed in a desiccator at 20 °C and 0 % RH (0 Pa water vapour pressure) containing silica. The cells were weighed at intervals of 2 h during 10 h. Steady-state and uniform water pressure conditions were assumed by keeping the air circulation constant outside the test cell by using a miniature fan inside the desiccator. The slope of weight loss versus time was obtained by linear regression. Three replicates were obtained for each sample.

4.2.9 Colour and opacity

The colour of the films was determined with a Minolta colorimeter (CR 400; Minolta, Japan). A white standard colour plate for the instruments' calibration was used as a background for colour measurements of the films, and the L^*, a^*, b^* values of each film were evaluated by reflectance measurements. In this system L^* indicates the lightness (ranging from black to white), and the horizontal axes, indicated by a^* and b^*, are the chromatic coordinates (ranging from – a^*: greenness, – b^*: blueness to + a^*: redness, + b^* yellowness). The values of a and b approach zero for neutral colours and increase as the colour becomes more chromatic and more saturated.

The opacity of a material is an indication of how much light passes through it: the higher the opacity, the lower the amount of light that can pass through the material. Generally, the opacity is calculated from reflectance measurements. The opacity of the samples was determined according to the Hunter lab method, as the relationship between the opacity of each sample on a black standard (Y_b) and the opacity of each sample on a white standard (Y_w) (Eq. 4-4). The measurements were repeated three times for each film.

$$Opacity = \frac{Y_b}{Y_w} \cdot 100 \qquad \text{Eq. 4-4}$$

4.2.10 Scanning electron microscopy (SEM)

The surface morphology of the films was examined using a scanning electron microscope (Nova NanoSEM 200, The Netherlands) with an accelerating voltage from 10 to 15 kV. Before analyses, all samples were mounted on aluminium stubs using carbon adhesive tape and sputter-coated with gold (thickness of about 10 nm).

4.2.11 Statistical analysis

Data analyses were performed using Microsoft Windows Excel 2003 and Statistica software (release 7, edition 2004, Statsoft, Tulsa, OK, USA).

The influence of the incorporation of the seeds extracts and the increase of galactomannan concentrations in galactomannan films were studied using a factorial design of 2^2 level with one centre point. The independent variables and their concentration range were: galactomannan concentration (X_1) from 0.5 to 1.5 % (w/v) and extract concentrations (X_2) from 0 to 1.0 % (w/v), where *Y* represents the dependent variables: *WVP*, Opacity, *TPC*, *RSA*, *L**, *a** and *b**.

The experimental data were fitted to a multifactor model, represented by Equation 4-5:

$$Y = a + b \cdot X_1 + c \cdot X_2 + d \cdot X_1 \cdot X_2 \qquad \text{Eq. 4-5}$$

The fitting accuracy of the model was evaluated by the calculation of the coefficient of determination (R^2) and the accuracy factor (A_f). The value of R^2, which gives the percentage of the variance of the data that is explained by the model, was calculated by:

$$R^2 = 1 - \frac{SSR}{SSD} \qquad \text{Eq. 4-6}$$

The higher the R^2 value, the better the model fits the experimental data (Neter, Kutner, Nachtsheim & Wasserman, 1996). The accuracy factor (A_f) provides information on the fitting accuracy. The closer the A_f value is to 1, the better the accuracy (Ross, 1996). A_f was calculated by:

$$A_f = 10^{\frac{\left|\sum_{j=1}^{j=J} \log(\frac{R_{pred}}{R_{obs}})\right|}{J}} \qquad j = 1, 2, 3, \ldots, j = \text{number of observations} \qquad \text{Eq. 4-7}$$

4.3. RESULTS AND DISCUSSION

4.3.1 Extraction yields of galactomannans and extracts

Table 4-2 presents the values from polysaccharide extraction yield (*PY*) showing that the pre-treatment can influence the final yield of the galactomannan extraction. Pre-treatment A (PTA) showed the highest value of *PY* (24 %), followed by pre-treatment B (PTB) (20 %) and pre-treatment C (PTC) (17 %). These differences may be explained by the fact that in PTA the seeds were only broken and not totally milled as in the other two pre-treatments. Further, the difference registered between the values of *PY* for PTB and PTC could be attributed to the 6 h of extraction used in PTC, when compared with the 15 min in PTB.

Table 4-2. Polysaccharide yield (*PY*), extract yield (*EY*), total phenolic content (*TPC*), Radical scavenging activity (*RSA*) and IC_{50} of the analysed extracts; *RSA* and IC_{50} values for BHT and BHA are given for comparison

Sample	*PY* (%)	*EY* (%)	*RSA* (%)	IC_{50} (mg mL^{-1})	*TPC* ($mg_{galic\ acid}\ g_{extract}^{-1}$)
A.1	24	0.04	18.77±3.22^{c}	13.31±0.67^{c}	6.13±0.69^{a}
A.3		0.07	71.59±3.60^{b}	1.40±0.37^{d}	10.79±0.99^{c}
B.1	20	0.14	61.88±5.32^{a}	3.94±0.49^{ab}	4.93±0.42^{a}
B.2		0.02	70.03±4.29^{b}	3.40±0.31^{a}	10.7±0.96^{bc}
B.3		0.02	71.11±4.42^{b}	3.20±0.18^{a}	9.90±0.81^{bc}
C.1	17	0.05	62.11±1.98^{a}	4.17±0.32^{b}	5.86±0.56^{a}
C.2		0.06	70.99±0.48^{b}	3.48±0.13^{a}	12.34±1.51^{c}
C.3		0.07	66.77±5.10^{ab}	3.45±0.28^{a}	9.82±0.33^{b}
BHT			76.36± 2.27^{d}	0.34±0.02^{e}	
BHA			76.91±3.17^{d}	0.06±0.01^{f}	

$^{a-f}$ Different superscript letters in the same column indicate a statistically significant difference (Tukey test $p<0.05$).

Extracts yield (*EY*) results are also shown in Table 4-2, being B.1 the extract with the highest *EY* value (0.14 %). The combination of the milling with a lower time of extraction, in the first step, provided higher values of *EY* for PTB, but in the following steps (B.2 and B.3) the values were lower when compared with PTA and PTC. The fraction A.3 has the second highest value of *EY*, as well as C.2 and C.3, which might be explained by the absence of the second extraction step. In this case, the water left during 24 h is used in the hydration of the galactomannan. The subsequent precipitation with ethanol thus includes the compounds that are not removed in step two as in the other treatments. Considering the total *EY* values, treatment B and C were the most efficient with a total value of 0.18 %, followed by the treatment A with total value of 0.11 %. Not surprisingly, in those pre-treatments where the milling of the seeds is one of the steps, the total *EY* was higher than in the pre-treatment where the seeds were only broken.

4.3.2 Determination of the total phenolic content (TPC)

The total phenolic content is given in Table 4-2. The extracts from the first step of the pre-treatments (A.1, B.1 and C.1) present the lowest values of *TPC*, with statistically significant difference ($p<0.05$) when compared with *TPC* values of the others steps (Table 4-2), being this explained by the ethanolic extraction performed in this step. This indicates that the phenolic compounds present in *G. triacanthos* extract are mostly constituted by water-soluble compounds. This fact is confirmed by the results for the extracts from step 2 (B.2 and C.2) that present the highest ($p<0.05$) value of *TPC*. In this step the seeds were left in water during 24 h. In PTA, the absence of step 2 makes of step 3 an extraction using water followed by ethanol, which explains the higher values ($p<0.05$) of *TPC* for extract A.3 when compared with A.1. Khokahar and Magnusdottir (2002) have reported water as the better solvent to the extraction of Zea polyphenols when compared with a mixture of 80 % of methanol and 70 % of ethanol. The obtained results are in agreement with the *TPC* values obtained to *Alpinia zerumbet* seeds that present values of 13.7 ± 0.4 $mg_{galic\ acid}\ g_{extract}^{-1}$ for extractions with diethyl ether (Elzaawely et al., 2007). However, they are lower than the *TPC* values obtained to guarana seeds that ranged between 119 and 186 $mg_{galic\ acid}\ g_{extract}^{-1}$ (Majhenič et al., 2007).

4.3.3 Antioxidant activity

The DPPH radical is widely used to evaluate the free-radical scavenging capacity of antioxidants (Zhenbao et al., 2007); in this work, it is presented in terms of the radical scavenging activity (*RSA*) and by IC_{50} that represent the concentration of the compounds that caused 50 % of inhibition of the oxidation. Therefore, the higher the value of *RSA* the lower will be the concentration necessary to cause 50 % of inhibition (IC_{50}). Table 4-2 shows that the highest ($p<0.05$) values of *RSA* were achieved by the fractions A.3, B.2, B.3, C.2 and C.3, being lowest value of IC_{50} found for the fraction A.3 ($p<0.05$). All the fractions corresponding to the first step of the pre-treatments (A.1, B.1 and C.1) have the lowest values ($p<0.05$) of *RSA* and, consequently, the highest values of IC_{50} confirming, in the case of fraction A.1, that milling the seeds before ethanol boiling in the pre-treatment process allows a more efficient antioxidant extraction thus increasing the antioxidant activity of those extracts. However, between fractions B.1 and C.1 no statistical significant differences could be observed ($p>0.05$), which leads to the conclusion that the time of extraction seems not to be an important parameter for antioxidant extraction. Fraction A.3 presents a significant antioxidant activity (and the lowest IC_{50}) when compared with the other fractions ($p<0.05$). This value is in agreement with the value of IC_{50} obtained for the water fraction from the methanolic extracts of *Cassia tora* L. seeds, where a value of 0.77 for IC_{50} (mg mL^{-1}) was reported (Zhenbao et al., 2007). *RSA* values are in agreement with the values obtained by Majhenič et al. (2007) in guarana seeds, with exception of fraction A.1, where the water and ethanolic extracts present 40 % higher values. The obtained values to IC_{50} are also in good agreement with other type of extracts. Kil et al. (2009) presented values of IC_{50} ranged between 4.0 and 129.0 mg mL^{-1} for 25 types of methanol sorghum extracts; and Subhasree et al. (2009) obtained values of IC_{50} ranged between 0.085 and 0.435 mg mL^{-1} to methanolic extracts of four plant species (*Trigonella foenum-graecum, Centella asiatica, Sauropus androgynus* and *Pisonia alba*).

A relationship between the *TPC*, *RSA* and IC_{50} is verified in Table 4-2, where higher values of *TPC* show a positive correlation with the antioxidant activity. The DPPH scavenging activity of flavonoids and phenolic acids has been studied in other works. Da Silva et al. (2006) showed that a positive correlation exists between the antioxidant activity of Brazilian commercial propolis extracts and their phenolic content. Hotta et al. (2002) proposed that the DPPH scavenging

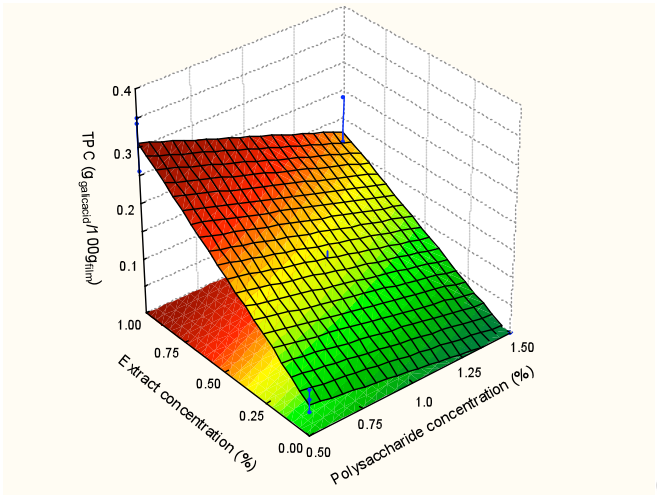
activity is related, not only to its H donation ability, but also to the subsequent polymerisation reaction. The extract selected to be used in film formulations was the one corresponding to the fraction A.3 where the best values of *TPC*, *RSA* and IC_{50} (10.79 $mg_{galic\ acid}\ g_{extract}^{-1}$, 71.59 % and 1.40 mg mL^{-1}, respectively) were obtained simultaneously.

4.3.4 Physicochemical properties of galactomannan films

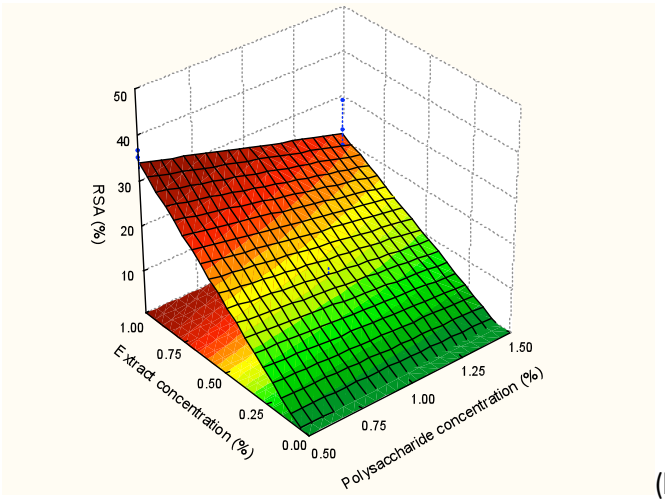
4.3.4.1 Total phenolic content (*TPC*) and radical scavenging activity (*RSA*)

The values of *TPC* and *RSA* in galactomannan films containing the extract as a function of the extract and galactomannan concentrations are shown in Figure 4-1a and 4-1b, respectively. As expected, these results demonstrate that galactomannan films with the incorporation of the extract present in both cases improved *TPC* and *RSA* when compared with the films without extract, due to the entrapment of the extract compounds in the film. The most significant factor affecting *TPC* and *RSA* in the studied films was the extract concentration (p<0.05, Table 4-3). The increase of *TPC* and *RSA* is generally proportional to the amount of extract added, with values ranged between 0.00 and 3.16 ± 0.51 $mg_{galic\ acid}\ g_{film}^{-1}$ to *TPC* and 0.00 and 36.17 ± 0.89 % to *RSA*. To films with 0.5 % of galactomannan and 0 % of extract (GT1) was detected a value 0.63 ± 0.19 $mg_{galic\ acid}\ g_{film}^{-1}$ to *TPC*. This value can be explained by the presence of phenolic compounds in the galactomannan polysaccharide that remain during the polysaccharide extraction process. The fitting of the model equation (Table 4-3) to the experimental values led to good results, with values of R^2 above 0.90 and A_f close to 1.

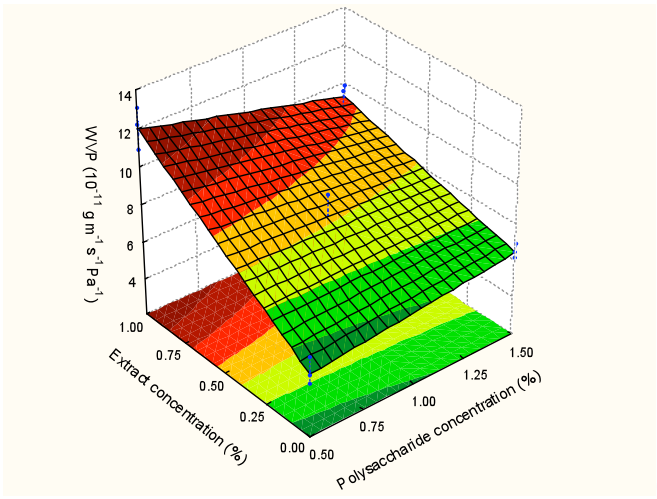
Figure 4-2 shows scanning electron microscopy (SEM) photographs of the films with and without extract incorporation. The film without extract shows a more uniform and compact structure, otherwise the film with extract shows granular vesicles, presumably due to the extracts entrapped in the film. The vesicles exhibit spherical shapes with smooth surfaces that are apparently free of visible cracks and pores. The obtained values of *TPC* and *RSA* for the galactomannan films showed that phenolic and antioxidant compounds added to the film forming solution can be later extracted from the galactomannan films. In the future, the releasing characteristic of antioxidants incorporated in the films should be investigated.



(a)



(b)



(c)

Figure 4-1. Values of total phenolic content (*TPC*) (a), radical scavenging activity (*RSA*) (b) and water vapour permeability (*WVP*) (c) in films as a function of both galactomannan and extract concentrations.

Table 4-3. Estimated values of the coefficients of Equation 4-5, calculated for the regression performed on: the water vapour permeability (*WVP*), total phenolic content (*TPC*) and radical scavenging activity (*RSA*), opacity, *L**, *a** and *b** values

Properties	Independent variable	Regression coefficient	Standard error	Significance level (*p*)	R^2	A_r
WVP	Constant	4.7542	0.7590	0.0001	0.91	1.00
	X_1	1.1268	0.6858	0.1286		
	X_2	8.5682	1.0843	0.0000		
	X_1X_2	-3.8637	0.9698	0.0021		
TPC	Constant	0.8631	0.3385	0.0270	0.92	0.92
	X_1	-0.6348	0.3058	0.0621		
	X_2	2.8728	0.4835	0.0001		
	X_1X_2	-0.6942	0.4325	0.1368		
RSA	Constant	-2.1236	4.9317	0.6751	0.90	0.83
	X_1	0.0000	4.4559	1.0000		
	X_2	42.5490	7.0453	0.0001		
	X_1X_2	-12.7641	6.3015	0.0678		
Opacity	Constant	5.8675	2.1450	0.0194	0.84	0.98
	X_1	3.3776	1.9380	0.1092		
	X_2	6.1494	3.0643	0.0700		
	X_1X_2	2.9432	2.7408	0.3059		
*L**	Constant	90.1641	1.9741	0.0000	0.95	0.99
	X_1	-1.8472	1.7836	0.3226		
	X_2	-20.1325	2.8201	0.0000		
	X_1X_2	1.3861	2.5224	0.5936		
*a**	Constant	4.7684	1.8182	0.0237	0.67	0.98
	X_1	0.1850	1.6427	0.9124		
	X_2	4.6592	2.5974	0.1003		
	X_1X_2	0.8183	2.3231	0.7313		
*b**	Constant	12.3195	5.9981	0.0645	0.93	0.96
	X_1	3.4517	5.4193	0.5372		
	X_2	57.3325	8.5687	0.0000		
	X_1X_2	-10.1239	7.6641	0.2133		

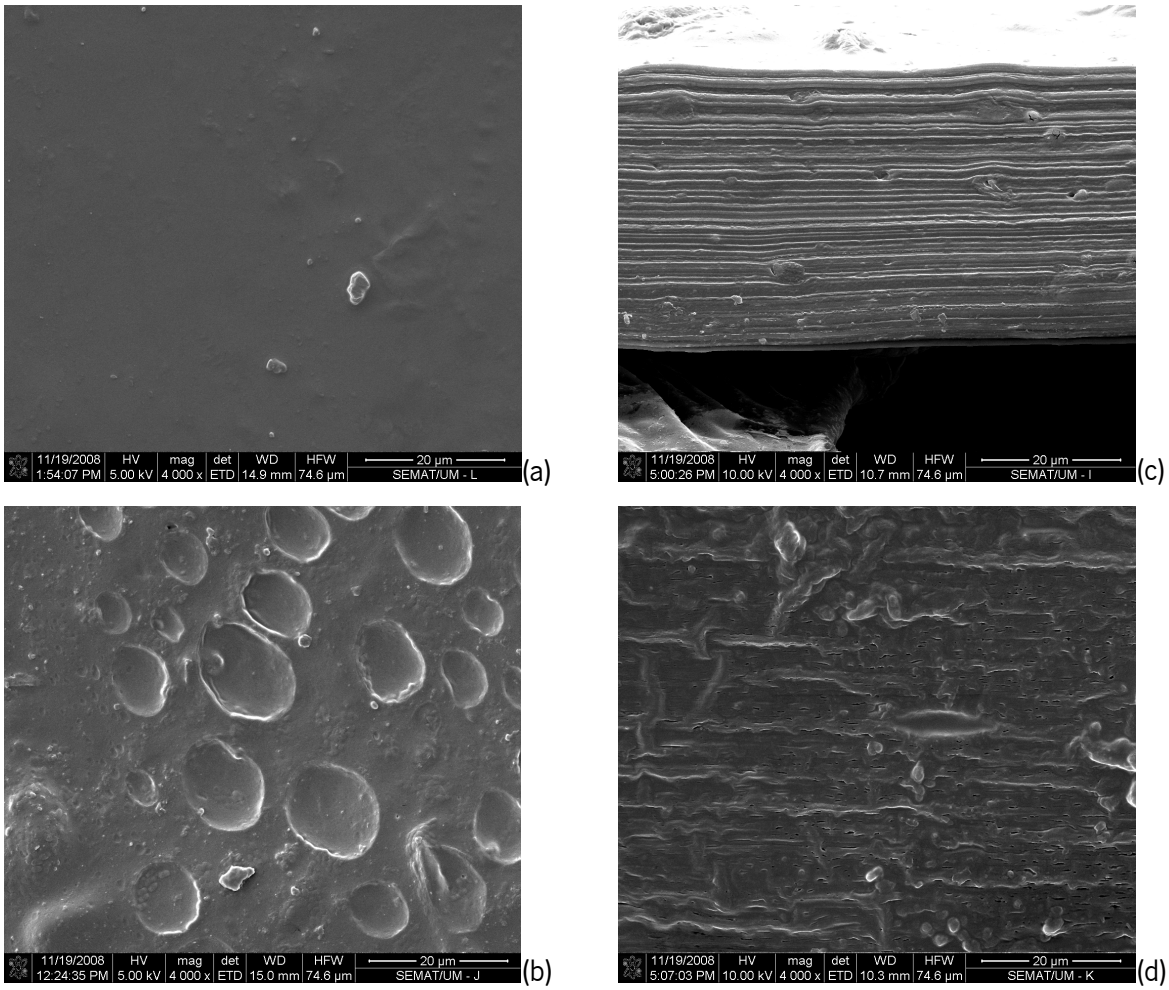


Figure 4-2. SEM images of surface (a and b) and cross-section (c and d) of galactomannan films without extract (a and c) and with extract (b and d) (magnification 4000 × and scale bar 20 μm).

4.3.4.2 Water vapour permeability

The water vapour permeability (*WVP*) is the most extensively studied property of edible films mainly because of the importance of the water in deteriorative reactions. Figure 4-1c shows the changes of *WVP* with the concentration of galactomannan and extract. The *WVP* values ranged from 5.02×10^{-11} (GT1) to 12.05×10^{-11} g m^{-1} s^{-1} Pa^{-1} (GT2), being significantly different between each other (p<0.05), and increasing with the increase of extract concentration (please see Table 4-4). The extract concentration and the interaction between the galactomannan and extract concentration are the most significant factors that affect the *WVP* (Table 4-3). The hydrophilic-

hydrophobic ratio of the film constituents plays an important role in the water vapour transfer process (Gómez-Estaca et al., 2009). Seed extracts contain phenolic acids and flavonoids that contain polar compounds, therefore contributing to improve the hydrophilic properties of the films thus increasing the *WVP*. Also the hydrophilic properties of the extracts can contribute to extend intermolecular interactions of the structural matrix in the galactomannan film, allowing the water vapour to pass through the film. Figure 4-2 shows that films without extract (Figure 4-2a and 4-2c) present a compact structure showing a higher cohesiveness that the films with extract, contributing to the decrease of *WVP*. Similar results were obtained by Nuthong et al. (2009) with porcine plasma protein-based films where the addition of tannic, caffeic and ferulic acids increased significantly the values of *WVP*.

The obtained values are in agreement with other published results for galactomannan films (Aydinli & Tutas, 2000). The *WVP* values obtained in the present work are comparable to tuna-fish gelatine based films containing *murta* extracts with values ranging between 5.08 and 7.97 × 10^{-11} g m^{-1} s^{-1} Pa^{-1} (Gómez-Guillén et al., 2007). The fitting of the model equation (Table 4-3) to the experimental values has shown good results, with values of R^2 above 0.91 and A_r very close to 1.

Table 4-4. Values of water vapour permeability (*WVP*), opacity, L^*, a^* and b^* of the films

Film	$WVP \times 10^{-11}$ (g m^{-1} s^{-1} Pa^{-1})	Opacity (%)	L^*	a^*	b^*
GT1	5.02±0.32^{a}	4.35±0.57^{a}	88.62±1.29^{a}	5.66±0.19^{a}	11.49±1.56^{a}
GT2	12.05±1.08^{b}	11.02±1.05^{b}	69.18±3.88^{b}	10.73±2.15^{b}	63.76±1.56^{b}
GT3	6.54±0.37^{c}	4.94±0.09^{a}	86.77±2.11^{ac}	5.85±0.68^{a}	14.94±2.20^{a}
GT4	9.31±0.67^{d}	20.09±1.68^{c}	68.72±0.28^{b}	11.73±0.15^{b}	57.09±4.12^{b}
GT5	7.86±1.16^{c}	16.89±0.80^{d}	81.42±2.30^{c}	4.49±1.77^{a}	49.59±4.01^{c}

$^{a-c}$ Different superscript letters in the same column indicate a statistically significant difference (Tukey test, $p<0.05$).

4.3.4.3 Colour and opacity

The opacity means a smaller transparency, important to control the incidence of light on food. Opacity values increase with the concentration of polysaccharide for all the studied films (Tab. 4-4). Also, the addition of extract to the films leads to an increase of the opacity values, essentially due to the colour of extract added. Also Gómez-Guillén et al. (2007) showed that the incorporation of *murta* extracts to tuna-fish gelatin films decrease the transparency of the resulting films. This increase is related with enrichment of films with phenol compounds and in some extends with the interaction between the phenol and polysaccharide (Gómez-Estaca et al., 2009). Table 4-4 shows the highest values ($p<0.05$) of a^* and b^* for the films containing extracts when compared with the films without extracts. In fact, the films with extract present a darker and yellower appearance as evidenced by the lower value of L^* and the higher values of b^*. Also Nuthong et al. (2009) for porcine plasma protein based films have shown a decreases in L^* value and an increases in a^* and b^* values, when the tannic and caffeic acids were added.

The films with extract can have advantages to due their higher opacity, which can affect the light transmission through the film, having better light barrier properties, which are advantageous when the light incidence is to be avoided. The extract concentration was the most significant factor (X_2) contributing to opacity and to the colour coordinates, but its statistical significance was detected only in L^* and b^* ($p<0.05$, Table 4-3). The fitting of the model equation (Table 4-3) to the experimental values has shown good results for L^* and b^* coordinates and not so good fitting to opacity and a^* values. This is reflected in the values of R^2 and A_r.

4.4 CONCLUSIONS

The results have shown that *G. triacanthos* seeds extracts can be used as a natural source of phenolic compounds and antioxidants; it was also shown that films produced from galactomannans of *G. triacanthos* are suitable to incorporate those antioxidants for further application in the food industry, while showing how the main film properties can change with galactomannan and antioxidant extract concentrations.

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CHAPTER 5

EFFECT OF GLYCEROL AND CORN OIL ON PHYSICOCHEMICAL PROPERTIES OF POLYSACCHARIDE COATINGS/FILMS

The influence of glycerol and corn oil on chitosan and galactomannan film properties was studied in this chapter. The influence of glycerol and oil on the film forming solutions was evaluated by the determination of the liquid-vapour interfacial tension and by the wettability in three different solid surfaces. Moisture content, solubility, water vapour permeability, mechanical properties and opacity were subsequently determined for the films resulting from the casting of the film forming solutions. The influence of glycerol and oil on films was also evaluated by Fourier transform infrared spectroscopy, differential scanning calorimetry and thermogravimetric analyses.

5.1 INTRODUCTION

The measurement of edible coatings/films properties (transport properties, opacity and mechanical properties) after their application on food can be very complex. This means that in most cases such determinations are made before application. For this purpose, coatings are obtained through solvent evaporation from film forming solutions which are casted in plates, where they form thin films that allow the measurement of their properties. These films can be representative of the coatings properties after application on food surface. The film forming solutions properties and their behaviour in different surfaces can be important since they can be related with the effectiveness of coatings application on food surfaces (Park, 1999). They must wet and spread the food surface, and after drying they should form a film (coating) with the adequate properties and durability. The surface properties of the food are a key factor in the process that involves wetting and coating (Hershko et al., 1996). Surface properties of the film forming solutions can also provide information about the phenomenon of wetting or non-wetting of a product's surface (Karbowiak et al., 2006). The surface energy properties are conditioned by: film homogeneity, the ability of the molecules to aggregate, the affinity of compounds for the solid support and the character of the solid support itself (Mirosław & Hołysz, 2010).

The application of edible coatings/films is limited by their high affinity to water when compared with synthetic/commercial plastics. The incorporation of other compounds, such as plasticizers and lipids, is common in order to improve mechanical and transport properties of edible coatings/films (Bergo & Sobral, 2007). However, it is necessary to determine their effects on coatings/films properties.

Plasticizers are commonly used to facilitate processing and/or to increase coatings/films flexibility. Water, oligosaccharides, polyols, and lipids are different types of plasticizers widely used in hydrocolloid-based films (Suyatma, 2005). Their combination could give rise to synergistic effects between components improving the properties of edible coatings/films. The lubrication theory postulates that plasticizers, by interspersing themselves, act as internal lubricants by reducing frictional forces between polymer chains. The gel theory postulates that the rigidity of the polymer comes from its three-dimensional structure, and plasticizers take effect

by breaking polymer-polymer interactions (e.g., hydrogen bonds and van der Waals or ionic forces). The free volume theory states plasticization as a way to increase free volume (Santosa & Padua, 1999; Suyatma, 2005). Glycerol is a major by-product of biodiesel production which has significantly increased, thus creating a significant surplus and is often regarded as a waste stream with an associated cost (Fountoulakis & Manios, 2009; Gu & Jérôme, 2010). The use of glycerol as plasticizer in these films can be a way to help solving the existing surplus of this co-product from biodiesel production. Lipids, due their hydrophobic behaviour, are added to polysaccharide films aiming at decreasing their hydrophilicity, consequently decreasing their water affinity (Vargas et al., 2009). From all the commercial oils, corn oil has shown to be the most effective, in comparison with others, in decreasing the water vapour permeability of polysaccharide and protein films (Ekthamasut & Akesowan, 2001; Tanaka et al., 2001).

One of the main disadvantages of biopolymers is that they naturally interact with water, leading to textural transformations that have a strong impact on their mechanical, transport and solubility properties. The presence of lipids and plasticizers can greatly influence the water content of polysaccharide films.

The aim of this study was to evaluate the influence of glycerol and corn oil presence on coatings/films properties. The liquid-vapour interfacial tension and the wettabillity of the coatings were evaluated for different glycerol and oil concentrations. These determinations were followed by analysing the resulting films by Fourier transform infrared spectroscopy, thermal analyses, solubility measurement, moisture content determination, water vapour permeability measurements, mechanical tests and opacity measurements.

5.2 MATERIALS AND METHODS

5.2.1 Film forming solutions and films preparation

Chitosan film forming solutions were prepared dissolving chitosan (deacetylation degree of 90 % approximately, Aqua Premier Co., Thailand) (1.5 % w/v) in a lactic acid (1.0 % v/v) solution with agitation using a magnetic stirrer (at 200 rpm) overnight at room temperature (20 °C); Tween 80

(0.2 %) was also added as surfactant. Galactomannan film forming solutions were prepared by dissolving galactomannan (1.5 % w/v) in distilled water, followed by the same conditions as for chitosan. Corn oil (Sovena, Portugal) was added in three different concentrations (0.25, 0.5 and 0.75 % w/v) with agitation during 20 min at 60 °C. Glycerol (87 %, Panreac, Spain) was added in three different concentrations (0.5, 1.25 and 2.0 % w/v). To produce the films, a constant amount (13 mL) of film forming solution was cast onto a 5.7 cm diameter Petri plate. The films were dried in an oven at 35 °C during 16 h. Films were maintained at 23 °C and 54 % RH at least 24 h before performing the tests (these conditions were obtained in a dessicator through a saturated salt solution of $Mg(NO_3)_2$ which was periodically replaced).

5.2.2 Wettability and surface tension

5.2.2.1 Critical surface tension and surface tension of selected solid surfaces

In systems having a surface tension lower than 100 mN m^{-1} (low-energy surfaces), the contact angle formed by a drop of liquid on a solid surface will be a linear function of the surface tension of the liquid, γ_{Lv}, (were phase *V* is air saturated with the vapour of liquid, *L*). Zisman's method (Zisman, 1964) is applicable only for low energy surfaces; that is the case of the three selected solid surfaces: glass, poly(methyl methacrylate) (PMMA) and stainless steel 316 (SS316) (Teixeira et al., 2005). For a pure liquid, if polar (γ_L^p) and dispersive (γ_L^d) interactions are known, and if θ is the contact angle between that liquid and a solid, the interaction can be described in terms of the reversible work of adhesion, *Wa*, as:

$$W_a = W_a^d + W_a^p \Leftrightarrow W_a = 2 \cdot \left(\sqrt{\gamma_S^d \cdot \gamma_L^d} + \sqrt{\gamma_S^p \cdot \gamma_L^p} \right) = \gamma_L \cdot (1 + \cos\theta) \qquad \text{Eq. 5-1}$$

where γ_s^p and γ_s^d are the polar and dispersive contributions of the surface of the studied solid. Rearranging Equation 5-1, yields:

$$\frac{1+\cos\theta}{2}\cdot\frac{\gamma_L}{\sqrt{\gamma_L^d}}=\sqrt{\gamma_S^p}\cdot\sqrt{\frac{\gamma_L^p}{\gamma_L^d}}+\sqrt{\gamma_S^d} \qquad \text{Eq. 5-2}$$

The contact angle determinations of at least three pure compounds: ethylene glycol (Merck, Germany), formamide (Merck, Germany) and ultra pure water, on the solid surface combined with the each dispersive and polar component values, will allow the calculation of both the independent variable $\left(\sqrt{\frac{\gamma_L^p}{\gamma_L^d}}\right)$, and the dependent variable, $\left(\frac{1+\cos\theta}{2}\cdot\frac{\gamma_L}{\sqrt{\gamma_L^d}}\right)_*$, from Equation 5-2.

5.2.2.2 Contact angle, wettability and liquid-vapour interfacial tension of film forming solutions

The wettability of the tested solutions was studied by determining the values of the spreading coefficient (W_s). The adhesive forces promote the liquid spreading in a solid surface and the cohesive forces promote their contraction. The wetting behaviour of the solutions will mainly depend on the balance between these forces. The surface tension of the coating solution was measured by the pendant drop method using the Laplace-Young approximation (Song & Springer, 1996).

The contact angle (θ) of a liquid drop on a solid surface is defined by the mechanical equilibrium of the drop under the action of three interfacial tensions: solid-vapour (γ_{SV}), solid-liquid (γ_{SL}), and liquid-vapour (γ_{LV}). The equilibrium spreading coefficient (W_s) is defined by Equation 5-3 (Rulon & Robert, 1993) and can only be negative or zero.

$$W_s = W_a - W_c = \gamma_{SV} - \gamma_{LV} - \gamma_{SL} \qquad \text{Eq. 5-3}$$

where W_a and W_c are the works of adhesion and cohesion.

Contact angle (θ) and liquid-vapour interfacial tension (γ_{LV}) were measured in a face contact angle meter (OCA 20, Dataphysics, Germany). The samples of the coatings were taken with a 500 μL syringe (Hamilton, Switzerland), with a needle of 0.75 mm of diameter. The contact angle at the solid surfaces was measured by the sessile drop method (Newman & Kwok, 1999), in which a droplet of the tested liquid was placed on a horizontal surface and observed with a face contact angle meter. Measurements were made in less than 5 s. Ten replicates of contact angle and surface tension measurements were obtained for each sample at 22.5 ± 0.5 °C.

The surfaces were chosen based in their similarity to the values obtained to food products as: fruits, vegetables and cheese (Hershko & Nussinovitch, 1998; Casariego et al., 2008; Martins et al., 2010). Their similarity allows to simulate the food surfaces and evaluate the influence of glycerol and oil on liquid-vapour interfacial tension of film forming solutions and also to show their effect on the wettability of the film forming solutions on three solid surfaces

5.2.3 Optical Microscopy

Optical observations of the film forming solutions were carried out with an optical microscope (Leitz Wetzlar, Germany) to which a digital camera was attached for image recording. The film forming solutions were evaluated using (×10) objectives and pictures were captured with Image Analysis software (Analysis get II).

5.2.4 Moisture Content

To determine the moisture content of films about 50 mg of film were dried at 105 °C during 24 h (until the equilibrium weight). The weight loss of the sample was determined, from which the moisture content was calculated using the following Equation:

$$Moisture\ content = \frac{(Mi - Mf)}{Mi} \cdot 100 \qquad \text{Eq. 5-4}$$

where M_i and M_f are the masses of initial and dried samples, respectively.

5.2.5 Fourier-transform infrared (FTIR) spectroscopy

The IR spectra of the films were determined using an infrared spectrometer (FTIR) (Perkin-Elmer 16 PC spectrometer, Boston, USA), in Attenuated Total Reflectance mode (ATR) between 400-4000 cm^{-1}, using 16 scans at a resolution of 4 cm^{-1}.

5.2.6 Differential scanning calorimetry (DSC) and thermogravimetric analyses (TGA)

Differential scanning calorimetry (DSC) measurements were performed with a Shimadzu DSC-50 (Shimadzu Corporation, Kyoto, Japan) calibrated with Indium as standard. Ca. 10 mg of the samples were placed in aluminium DSC pans (Al crimp Pan C.201-52943). The measurements were performed between -100 and 250 °C at a heating rate of 10 °C min^{-1} under a nitrogen atmosphere. In a first heating scan the enthalpy of melting (ΔH_m) and the melting peak (T_m) were determined; a second heating allows the measurement of the glass transition temperature (T_g). Thermogravimetric analyses (TGA) were completed with a Shimadzu TGA-50 (Shimadzu Corporation, Kyoto, Japan). Samples were placed in the balance system and heated from 20 °C to 580 °C at a heating rate of 10 °C min^{-1} under a nitrogen atmosphere.

5.2.7 Water solubility

The films' solubility in water was determined according to the method reported by (Cuq et al., 1996). Solubility is defined as the content of dry matter solubilised after 24 h immersion in

water. The initial dry matter content of each film was determined by drying to constant weight in an oven at 105 °C. A disk of film (2 cm diameter) were cut, weighed (M_i), and immersed in 50 mL of water. After 24 h of immersion at 20 °C with agitation (60 rpm), the pieces of film were taken out and dried to constant weight (M_f) in an oven at 105 °C, to determine the weight of dry matter that was not solubilised in water. The solubility of the films was then determined as follows:

$$Water\ Solubility = \frac{(Mi - Mf)}{Mi} \cdot 100 \qquad \text{Eq. 5-5}$$

where M_i is the initial mass and M_f is the final mass of the sample.

5.2.8 Film thickness

Film thickness was performed as explained in Chapter 4 (section 4.2.7). Mean values were used to calculate gas permeability and mechanical properties.

*5.2.9 Water vapour permeability (*WVP*)* measurement

The measurement of water vapour permeability (*WVP*) was performed as explained in Chapter 4 (section 4.2.8).

*5.2.10 Tensile strength (*TS*) and elongation-at-break (*EB*)*

TS and *EB* were measured with an Instron Universal Testing Machine (Model 4500, Instron Corporation) following the guidelines of ASTM Standard Method D 882-91. The initial grip separation was set at 30 mm and the crosshead speed was set at 5 mm min^{-1}. *TS* was expressed in Pa and calculated by dividing the maximum load (N) by the initial cross-sectional area (m^2) of the specimen. *EB* was calculated as the ratio of the final length at the point of sample rupture to the initial length of a specimen (30 mm) and expressed as a percentage. According to the ASTM standard, film strips with a length of 45 mm and a width of 20 mm were used. *TS* and *EB* tests were replicated at least three times for each type of film.

5.2.11 Opacity

The opacity of the samples was determined as explained in Chapter 4 (section 4.2.9).

5.2.12 Statistical analyses

Statistical analyses were performed using Analysis of Variance (ANOVA) and linear regression analysis. The Tukey test (α=0.05) was used to determine any significance of differences between specific means (SigmaStat, trial version, 2003, USA).

5.3 RESULTS AND DISCUSSION

5.3.1 Water contact angle and surface tension of selected solid surfaces

The interaction between the solid surface and a liquid is given by the contact angle value. A high contact angle corresponds to a weak interaction between water and the solid surface. In contrast, a low contact angle indicates a strong interaction between water and the solid surface. The

interaction between water and the solid surfaces is related to the polar and apolar components of the surface tension of the surfaces (Gülec et al., 2006).

Table 5-1 shows the contact angle between water and the studied surfaces, and the surface tension (polar and dispersive components) of the surfaces. The values of the dispersive component of glass and PMAA surfaces do not present a statistically significant difference (p>0.05) between them, and are statistically different (p>0.05) from the dispersive component of SS316. For the polar component the values increase for the three studied materials in the following order: SS316 < glass < PMMA. Results show that PMMA presents a higher affinity to interact with polar compounds, as demonstrated by the contact angle value between water and PMMA (Table 5-1). These results are in agreement with the behaviour reported in the literature, where glass has been described to present a more hydrophilic behaviour than SS316 (Gülec et al., 2006).

Table 5-1. Values of water contact angle, surface tension, polar and dispersive components of the surfaces of selected materials

Material	Contact angle (θ)	Surface tension (mN m^{-1})	Polar component (mN m^{-1})	Dispersive component (mN m^{-1})
SS316	62.15±3.82^{a}	45.28±0.16^{a}	9.40±0.10^{a}	35.88±0.13^{a}
Glass	59.88±4.13^{ab}	48.26±0.05^{b}	12.21±0.03^{b}	36.05±0.04^{b}
PMMA	54.15±4.71^{b}	50.33±0.10^{c}	14.21±0.06^{c}	36.12±0.08^{b}

$^{a–c}$ Means in the same column with different superscripts are significantly different (p<0.05).

5.3.2 Chitosan film forming solutions

5.3.2.1 Wettability

The knowledge of the wettability of a film forming solutions is of particular importance as it is a parameter that defines the ability of a coating to be uniformly distributed on the food surface (Lin & Zhao, 2007).

Glycerol and corn oil were added to the film forming solutions in order to evaluate their influence in the liquid-vapour interfacial tension (γ_{LV}) of film forming solutions and also to show their effect on the wettability of the film forming solutions on three solid surfaces. Film forming solutions properties and their behaviour in different solid surfaces can be important since they can be related with the effectiveness of coating solutions when applied on different food products (Park, 1999).

Table 5-2 shows the liquid-vapour interfacial tension (γ_{LV}) values for chitosan film forming solutions. The results show that the increase of glycerol concentration leads to a decrease of the γ_{LV} values. This behaviour can be explained by the increase of the surface pressure of the film forming solutions, that decreases the liquid-vapour interface (Choi et al., 2002). The obtained values are in agreement with the values reported for chitosan film forming solutions (Choi et al., 2002; Ribeiro et al., 2007; Vargas et al., 2009). The solvent of the film forming solutions presents a liquid-vapour interfacial tension (results not shown) higher than that of the film forming solutions themselves; this liquid-vapour interfacial tension decreases with the incorporation of chitosan and with the increase of glycerol concentrations. Also the presence of corn oil in the chitosan film forming solutions leads to a decrease of liquid-vapour interfacial tension values (Table 5-3). The results obtained are in agreement with those reported for chitosan and sodium caseinate film forming solutions, where it has been shown that the presence of oleic acid decreases the liquid-vapour interfacial tension of the corresponding film forming solutions (Fabra et al., 2009; Vargas et al., 2009).

Table 5-2 and Table 5-3 show the values of the spreading coefficient (W_s) of the film forming solution on the solid surfaces. In practical terms, the closer the W_s values are from zero, the better the surface will be coated. The polar component of the solid surfaces appears to influence the obtained W_s values. SS316 presents the lowest polar component and the highest W_s values when compared with the other surfaces. Therefore, the solid with the highest polar (p<0.05) component value (PMMA) presents the highest affinity with the film forming solutions.

The increase of glycerol concentrations leads to lower values of W_s for all the solid surfaces (Table 5-2). The increase of glycerol concentrations leads to a change of the film forming solution behaviour towards a greater affinity to hydrophilic surfaces. Film forming solutions with higher

concentrations of glycerol (2.0 %) present W_s values which are closer to each other for all studied samples.

Table 5-2. Values of liquid-vapour interfacial tension (γ_{LV}) and spreading coefficient (W_s) on the solid surfaces of the film forming solutions of 1.5 % chitosan for increasing glycerol concentrations

Glycerol % (w/v)	γ_{LV} (mN m^{-1})	W_s (mN m^{-1})		
		PMMA	Glass	SS316
0	37.32±0.65^{a}	-11.74±1.02$^{a;A}$	-16.12±1.34$^{a;B}$	-21.71±1.43$^{a;C}$
0.5	36.45±0.64^{ab}	-11.00±1.39$^{a;A}$	-13.39±0.88$^{b;B}$	-19.13±1.43$^{b;C}$
1.25	35.47±0.61^{b}	-9.48±0.27$^{b;A}$	-10.68±1.07$^{c;AB}$	-12.11±1.09$^{c;B}$
2	32.90±0.57^{c}	-8.32±0.44$^{c;A}$	-9.35±0.66$^{c;A}$	-11.17±1.06$^{c;B}$

$^{a–c,A-C}$ Means in the same column with different superscripts (lower case) are significantly different (p<0.05). For the W_s values means in the same line with different superscripts (upper case) are significantly different (p<0.05).

Table 5-3. Values of liquid-vapour interfacial tension (γ_{LV}) and spreading coefficient (W_s) on the solid surfaces of the film forming solutions of 1.5 % chitosan and 0.5 % of glycerol for increasing oil concentrations

Oil % (w/v)	γ_{LV} (mN m^{-1})	W_s (mN m^{-1})		
		PMMA	Glass	SS316
0	36.45±0.64^{a}	-11.00±1.39$^{a;A}$	-13.39±0.88$^{a;B}$	-19.13±1.43$^{a;C}$
0.25	31.28±0.54^{b}	-11.76±1.27$^{a;A}$	-10.60±0.77$^{b;A}$	-11.22±0.42$^{b;A}$
0.5	30.34±0.53^{b}	-9.41±0.71$^{b;A}$	-9.79±0.56$^{bc;A}$	-10.85±0.57$^{bc;B}$
0.75	28.54±0.50^{c}	-8.44±1.01$^{b;A}$	-8.31±0.93$^{c;A}$	-8.95±1.25$^{c;A}$

$^{a–c,A-C}$ Means in the same column with different superscripts (lower case) are significantly different (p<0.05). For the W_s values means in the same line with different superscripts (upper case) are significantly different (p<0.05).

Corn oil presence leads to a decrease of the W_s values between the film forming solutions and the solid surfaces (Table 5-3). It can also be expected that the hydrophobic character of oil promotes its interaction with the dispersive component of the solid surfaces. Table 5-3 shows that W_s values decreased when 0.25 % of oil was added to the film forming solutions. The presence of oil in the film forming solutions leads to very similar W_s values (p>0.05) for the three solid surfaces. This behaviour can be related with the dispersive components of the solids that present similar values (Table 5-1), which overlap the differences registered for the respective polar components. The dispersive component of the solid surfaces has a high influence on W_s values when oil was added to the film forming solutions, reducing the differences of W_s values between the solid surfaces. It is remarkable that the presence of glycerol and corn oil decrease the W_s of the film forming solutions on the studied surfaces.

Figure 5-1 shows microscopy images of the film forming solutions without oil (Figure 5-1a) and with increasing oil concentrations (Fig. 5-1(b, c and d).

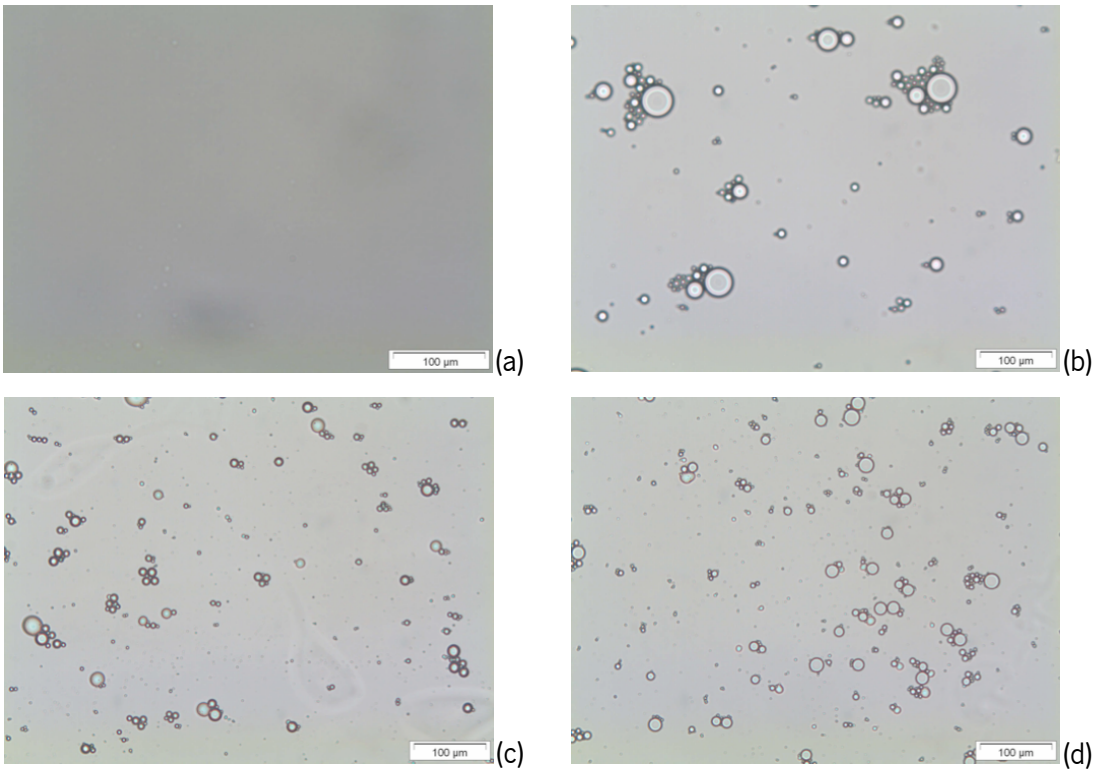

Figure 5-1. Optical microscopy images (100 ×) of chitosan film forming solutions for: 1.5 % of chitosan with 0.5 % of glycerol: without oil (a); 0.25 % of oil (b); 0.5 % of oil (c) and 0.75 % of oil (d).

The presence of Tween 80 leads to the formation of lipid micelles in the chitosan film forming solutions. The study of the lipid particle size can be important to better understand the influence and the distribution of oil particles in the film forming solutions. The size of the oil particles ranged from 2 to 50 μm, in the range of other reported values for film forming solutions with lipids (Fabra et al., 2009; Sánchez-González et al., 2009; Vargas et al., 2009). It was observed that for higher oil concentrations the size of the particles decreased and the number of particles increased. Figure 5-1 also shows the presence of aggregates of lipid micelles that can influence the distribution of the lipid phase in the film forming solutions.

5.3.2.2 Fourier-transform infrared (FTIR) spectroscopy and moisture content

The effect of glycerol and oil in chitosan films was initially evaluated by FTIR and by the moisture content of the films. Figure 5-2 shows the FTIR spectra of the chitosan films containing 0, 0.5, 1.25 and 2.0 % of glycerol. The broad band ranging between 3500 and 3100 cm^{-1} is attributed to O-H stretching vibration that overlaps the N-H stretching vibration in the same region. The broad band between 2800 and 3000 cm^{-1} is attributed to C-H stretching vibration. The peak at 1574 cm^{-1} was due to the N-H bending (amide II); and the peak at 1733 cm^{-1} suggests the presence of a carbonyl group (C=O) in the film matrix (Xu et al, 2005; Ziani et al., 2008). When compounds are mixed, physical blends and chemical interactions are reflected by changes in characteristic spectra peaks (Xu et al., 2005). Glycerol incorporation leads to the presence of new bands at 998 cm^{-1} and 925 cm^{-1} (being more intense for concentrations of 1.25 % and 2.0 % of glycerol) that correspond to asymmetric and symmetric stretching vibrations of the alcoxyl group (C-O-C), respectively (Jamróz et al., 2007). Figure 5-2 shows that for higher glycerol concentrations the intensity of the broad band that corresponds to the O-H scretching vibration increases. This behaviour is explained by the increase of water content in the film matrix for higher glycerol concentrations, as confirmed by the moisture content of the chitosan films (Figure 5-3).

Figure 5-3 and Figure 5-4 show the variations of the moisture content in chitosan films for increasing concentrations of glycerol and oil, respectively. The presence of water in chitosan films is highly dependent of the glycerol concentration. Glycerol, due to its hydrophilic nature, retains water in the film matrix. Higher concentrations of plasticizer favour the adsorption of water molecules, which is mainly attributed to the predisposition of plasticizers to form hydrogen

bonds. On the other hand, it is observed that moisture content decreases for films with oil (Figure 5-4). However, the values do not present a statistically significant difference (p>0.05) between the film samples with oil.

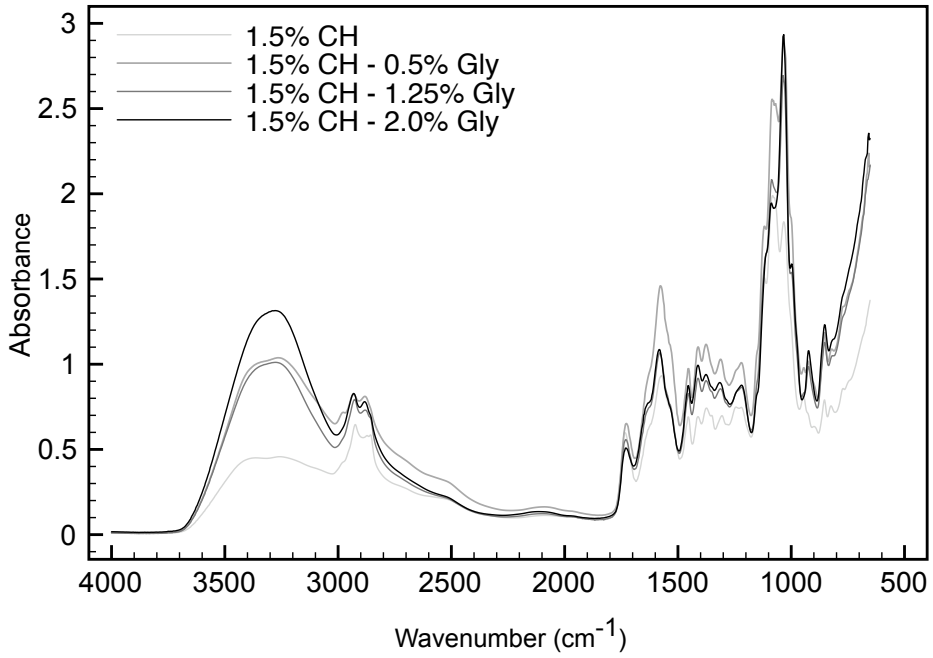

Figure 5-2. FTIR spectra of chitosan films for increasing glycerol concentrations.

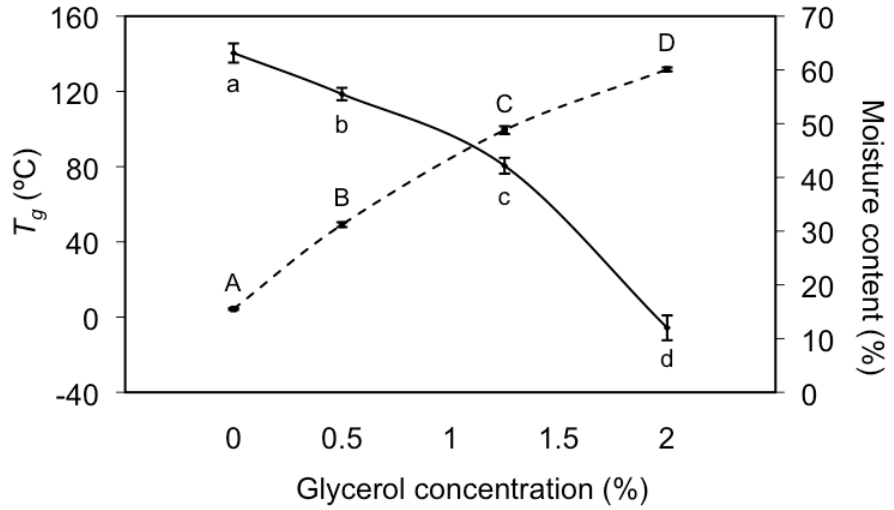

Figure 5-3. Glass transition temperature (T_g) (—) and moisture content (- - -) of chitosan films for increasing glycerol concentrations. [a–d;A–D]Means with different superscripts are significantly different (p<0.05).

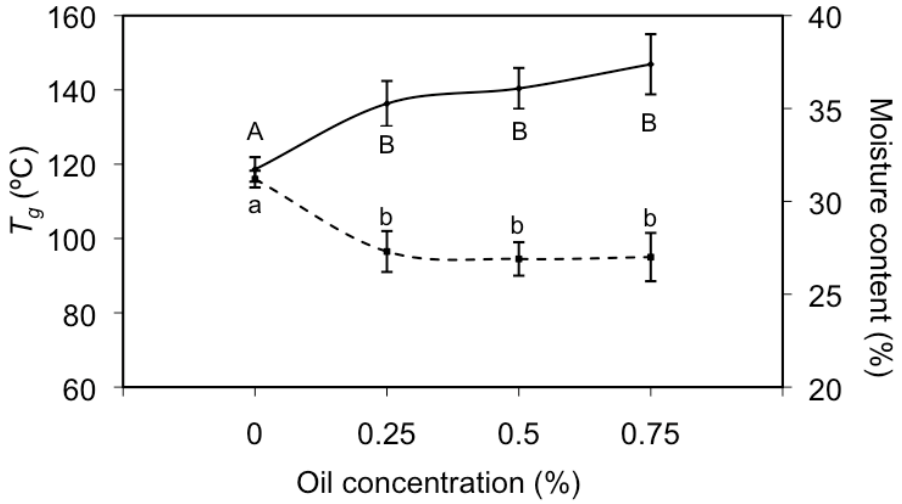

Figure 5-4. Glass transition temperature (T_g) (—) and moisture content (- - -) of chitosan films for increasing oil concentrations. $^{a-b;A-B}$Means with different superscripts are significantly different (p<0.05).

Figure 5-5 shows the FTIR spectra of chitosan films for increasing oil concentrations. The oil incorporation lead to a change of the bands corresponding to 1726 cm^{-1} that shifted to 1732 cm^{-1} and 1742 cm^{-1} for chitosan films with 0.5 % and 0.75 % of oil, respectively. This difference in the stretching vibration, that corresponds to the C=O, can be explained by the presence of the ester carbonyl functional group of the triglycerides, associated with the corn oil at 1746 cm^{-1} (Vlachos et al., 2006). FTIR spectra of chitosan films with 0.75 % of oil also show a high and more intense number of peaks in the frequency range between 2800 and 3100 cm^{-1}. The band near to 3009 cm^{-1} is associated with the C-H stretching vibration of the *cis*-double bond of the corn oil fatty acids; and the peak at 2925 cm^{-1} can be related with the symmetric and asymmetric stretching vibration of the aliphatic group (CH_2) (Vlachos et al., 2006).

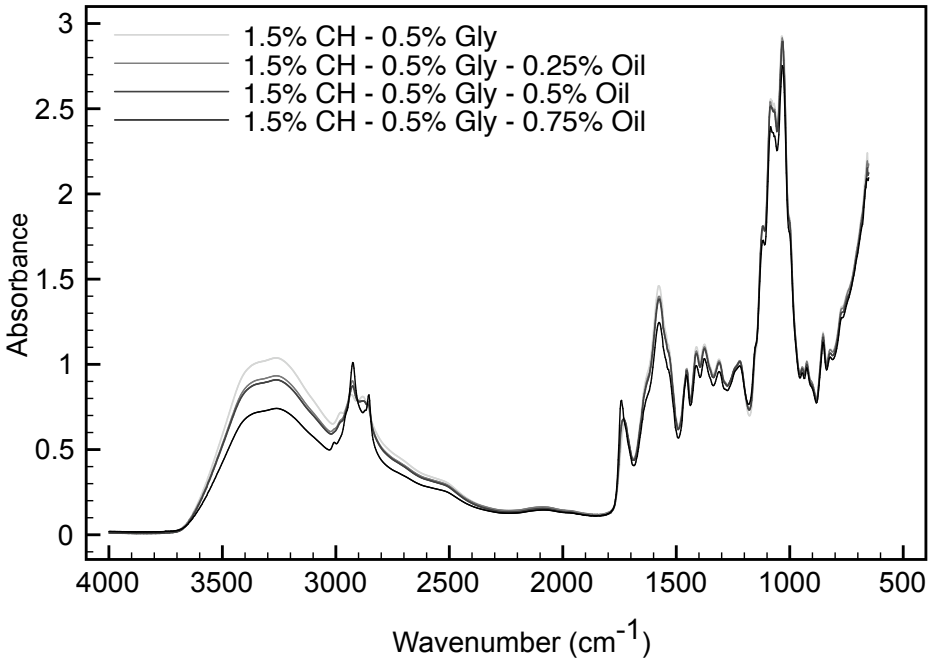

Figure 5-5. FTIR spectra of chitosan (CH) films for increasing oil concentrations.

5.3.2.3 Thermal analyses

Differential scanning calorimetry (DSC) analyses were used to measure the glass transition temperature (T_g), the enthalpy of melting (ΔH_m) and the peak melting temperature (T_m) of the films. T_g is a parameter associated with the system mobility, and is defined as a physical change from the glassy to the rubbery state in amorphous materials promoted by heat (Roos & Karel, 1991). Figure 5-3 shows T_g values for chitosan films with different glycerol concentrations. Glycerol, due its plasticizer effect, decreases the glass transition temperatures of polysaccharides, which is in agreement with the free volume theory of plasticization. The increase of glycerol concentration leads to an increase of the free volume and mobility of molecules, changing the physical structure of the chitosan film, which is in agreement with the decrease of the T_g values. Moreover, T_g values are inversely associated with the moisture content of the chitosan films; in fact, also water acts as a plasticizer increasing the molecular mobility (lower T_g values) of the chitosan films. Glycerol changes the polymer network creating mobile regions with greater interchain distances, promoting water clustering (Diab et al., 2001; Olivas & Barbosa-Cánovas, 2008), thus increasing the moisture content in the films. The thermograms present a

great decrease of T_g values for glycerol concentrations of 2.0 %, that can be explained by the lower value reported for the T_g of glycerol (-75 °C) (Mathew & Dufresne, 2002). T_g values obtained here are in agreement with those reported in other works (Dong et al., 2004; Suyatma, 2005).

Oil incorporation decreases the mobility of the chitosan matrix, as confirmed by the increase of T_g values (Figure 5-4). However, the increase of oil concentrations does not lead to statistically significant differencees (p>0.05) between T_g values. This behaviour can be explained by the moisture content of the films containing oil, that also does not present statistically significant difference (p>0.05).

Figure 5-6 shows that the increase of glycerol concentrations leads to an increase of ΔH_m, and to a decrease of T_m values. The higher values of ΔH_m can be explained by the increase of the crystallinity of chitosan films. When glycerol concentration increases, a greater polymer mobility (lower T_g values) is obtained that favours the formation of crystalline domains (Mathew et al., 2002; Fabra et al., 2010). Also the increase of the moisture content in the film matrix when more hydrogen bonds are available can influence the intensification of films' crystallinity (Chen et al., 2008). The presence of oil does not provoke statistically significantly differences (p>0.05) on the values of ΔH_m and T_m (results not shown).

Table 5-4 shows the peak values of thermal events and the corresponding weight loss. Thermal analyses show that chitosan films began the dehydration process at 60 °C (results not shown), being stable below that temperature. They present at least three thermal events, however for samples with oil a fourth event was observed. Peak 1 is related with the evaporation process, a characteristic phenomenon of a polysaccharide with a hydrophilic nature. The differences in weight loss due to the presence of glycerol (peak 2) are also very marked, where an increase of the weight loss for higher glycerol concentrations is observed, related with the loss of chemisorbed water through hydrogen bonds and the elimination reaction of NH_3 (Quijada-Garrido et al., 2007). Peak 3 (around 290 °C) is related with the dehydration, depolymerization and pyrolytic decomposition of the polysaccharide backbone (Zohuriaan & Shokrolahi, 2004).

In films containing oil, where the plasticizer content is constant, the weight loss at peak 2 (related with the presence of glycerol) does not present statistically significant differences; however, there is an increase in the peak associated with oil decomposition (peak 4), related with the aromatic structures present in corn oil with decomposition temperatures above 380 °C (Pelissari et al., 2009).

Table 5-4. Thermal behaviour of chitosan films. The values of the peaks correspond to the values of the derivative thermograms obtained from the TGA curve

Films* 1.5 % CH	Peak 1 (°C)	Weight loss (%)	Peak 2 (°C)	Weight loss (%)	Peak 3 (°C)	Weight loss (%)	Peak 4 (°C)	Weight loss (%)
-	99.1	-10.1	207.7	-13.1	299.2	-37.2	-	-
0.5 % Gly	66.0	-11.3	195.6	-28.2	295.4	-30.3	-	-
1.25 % Gly	71.8	-12.1	200.4	-33.1	293.2	-28.6	-	-
2.0 % Gly	63.6	-14.2	201.6	-37.1	292.3	-26.2	-	-
0.5 % Gly-0.25 % Oil	98.8	-10.3	190.1	-24.8	296.2	-30.2	429.8	-6.1
0.5 % Gly-0.5 % Oil	75.7	-9.9	203.4	-27.6	294.2	-32.1	427.1	-8.5
0.5 % Gly-0.75 % Oil	78.0	-7.1	200.6	-25.3	290.9	-29.8	423.9	-13.8

* Films with 1.5 % of chitosan (CH) with increasing concentrations of glycerol (Gly) and oil.

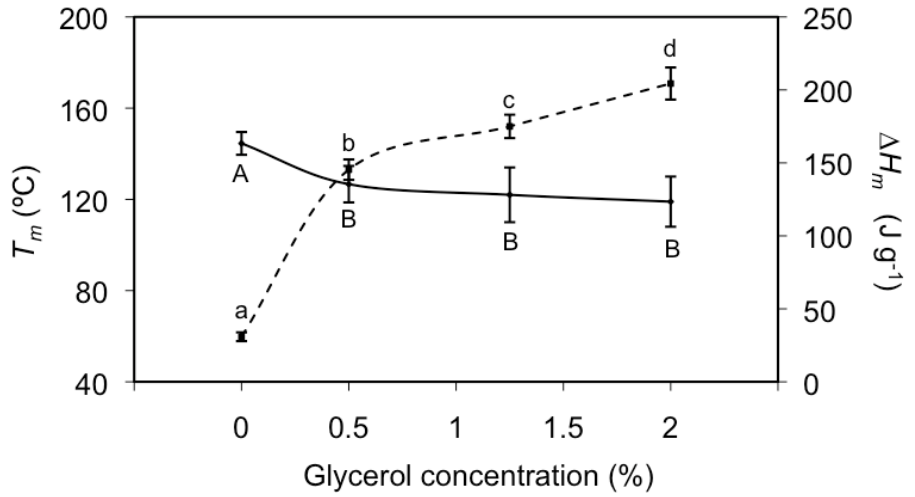

Figure 5-6. Melting temperature peak (T_m) (—) and enthalpy of melting (ΔH_m) (- - -) of chitosan films for increasing glycerol concentrations. $^{a-d;A-B}$Means with different superscripts are significantly different (p<0.05).

5.3.2.4 Water solubility

The water solubility of edible films indicates their water resistance when applied, e.g., on food. It is also related to the biodegradability of films when used as packaging materials (Gnanasambadam et al., 1997). Figure 5-7 shows that water solubility increases for higher glycerol concentrations (p<0.05). This is related with the hydrophilic behaviour of glycerol and with the O-H bonds of chitosan films that are more available to interact with the water molecules. On the other hand, the presence of oil and the increase of its concentration lead to a statistically significant decrease (p<0.05) of the solubility. The decrease of the number of O-H bonds, the appearance of the aliphatic groups when oil is added, and the increase of the hydrophobic portion of the film originated a less soluble material.

The values obtained are in agreement with the reported values of solubility for chitosan films (Casariego et al., 2009; Souza et al., 2009).

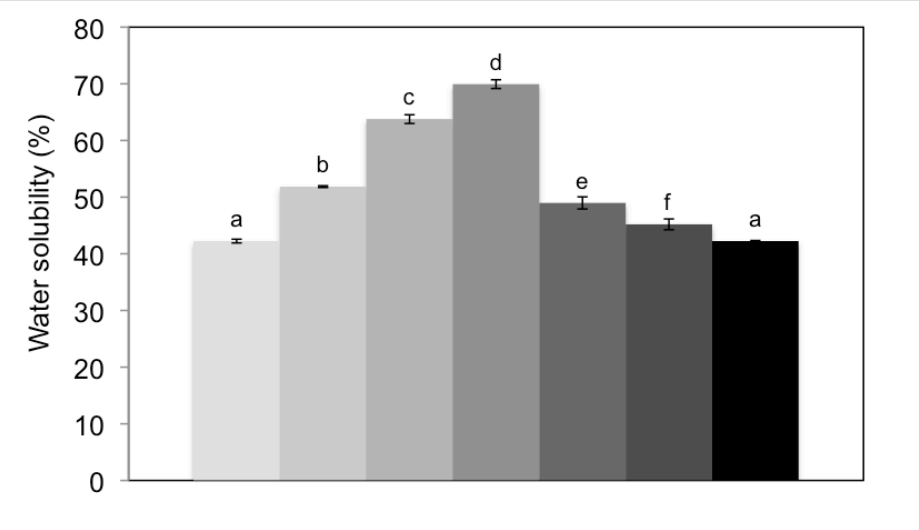

Figure 5-7. Water solubility of chitosan (CH) films for increasing glycerol (Gly) and oil concentrations (1.5 % CH ; 1.5 % CH – 0.5 % Gly ; 1.5 % CH – 1.25 % Gly ; 1.5 % CH – 2.0 % Gly ; 1.5 % CH – 0.5 % Gly – 0.25 % Oil ; 1.5 % CH – 0.5 % Gly – 0.5 % Oil ; 1.5 % CH – 0.5 % Gly – 0.75 % Oil . [a-f]Means with different superscripts are significantly different (p<0.05).

5.3.2.5 Water vapour permeability (*WVP*)

The water vapour permeability is the most extensively studied property of edible films. Figure 5-8 shows the influence of glycerol and oil in the *WVP* values. Oil and glycerol show a distinct influence in *WVP* of chitosan films; while the *WVP* values increase for increasing glycerol concentrations, the presence of oil leads to a decrease of *WVP*. These results are in agreement with other works where the increase of plasticizer concentration has increased the values of *WVP* (Caner et al., 1998; Ziani et al., 2008). As already explained above, the increase of glycerol concentration leads to higher moisture contents of chitosan films. The plasticizer action increases the free volume and chain movements (lower T_g), reducing the rigidity and increasing the molecular mobility of films, thus allowing a higher water vapour diffusion.

As already reported, the incorporation of lipids can be used to decrease the *WVP* values of polysaccharide films (Wong et al., 1992; Park & Zhao, 2004; Vargas et al., 2009). The presence

of oil changes the film's properties, decreasing the affinity for water. The decrease of the *WVP* values with the addition of oil can be explained by the diminution of the hydrophilic portion of the film (Hernandez-Munõz et al., 2004), that reduces its affinity for water molecules and consequently decreases *WVP* (Figure 5-8). As already stated, higher glycerol concentrations lead to an increase of the moisture content in films, while for different oil concentrations the moisture content values do not present statistically significant differences (p>0.05). So, if in the case of films without oil the glycerol effect was emphasised by the water influence, for films with oil this does not happen. Being so, the oil presence is the principal responsible for the decrease of *WVP* values. The range of values obtained is in agreement with other reported works (Wong et al., 1992; Casariego et al., 2008).

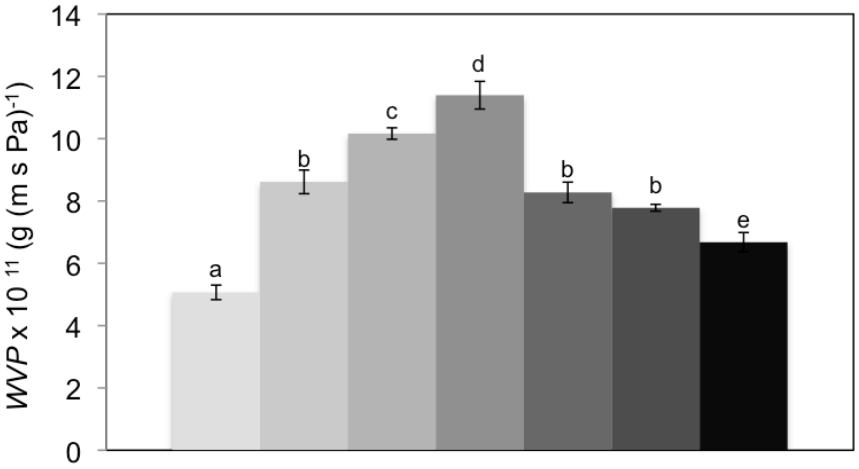

Figure 5-8. Water vapour permeability (*WVP*) of chitosan (CH) films for increasing glycerol (Gly) and oil concentrations (1.5 % CH ; 1.5 % CH – 0.5 % Gly ; 1.5 % CH – 1.25 % Gly ; 1.5 % CH – 2.0 % Gly ; 1.5 % CH – 0.5 % Gly – 0.25 % Oil ; 1.5 % CH – 0.5 % Gly – 0.5 % Oil ; 1.5 % CH – 0.5 % Gly – 0.75 % Oil . [a–e] Means with different superscripts are significantly different (p<0.05).

5.3.2.6 Tensile strength (*TS*) and elongation-at-break (*EB*)

Tensile strength is the ability of a material to resist under tensile stress until break and is one of the most important and widely measured properties of materials used in structural applications. Elongation-at-break of an engineering material is the percentage increase in length that occurs before it breaks under tension (Sperling, 2006).

The increase of plasticizer concentration exerts a great influence over *TS* values, and lead to a decrease of approximately 64 % when 0.5 % of glycerol were added (Figure 5-9). For the same amount of added glycerol, *EB* values were 4.4-fold those of chitosan films without glicerol. Furthermore, when glycerol concentration increased from 0.5 % to 2.0 %, this behaviour was more evident with a decrease of *TS* values of approximately 85 % and an increase of *EB* values of 39 %. Plasticizers interfere with chitosan chains: they decrease intermolecular forces, soften the rigidity of the film's structure and increase polymer mobility (in agreement with the T_g values observed), thus decreasing *TS* and increasing *EB*. The presence of glycerol leads to a ductile and flexible material. Furthermore, also the water content of the films when glycerol is added affects *TS* and *EB*, and accentuates the effect of the glycerol content (Ziani et al., 2008). These results are in agreement with those reported in the literature that show a decrease of *TS* values and an increase of *EB* values with the presence and increasing concentrations of plasticizer (Caner et al., 1998; Ziani et al., 2008).

Figure 5-10 shows that the presence of oil influences both *TS* and *EB* values. Oil incorporation leads to a decrease of *TS* and *EB* values when compared with the films without oil. Increasing oil concentrations from 0.5 to 0.75 % did not have statistically significant influences ($p>0.05$) on *TS* and *EB* values for chitosan films ($p>0.05$), suggesting that the chitosan matrix is not able to absorb oil concentrations above 0.5 %. The oil presence leads to a less rigid film structure, being the structural discontinuities provoked by the oil incorporation possibly responsible for the decrease of their flexibility and their resistance to fracture (Sánches-Gonzáles et al., 2009).

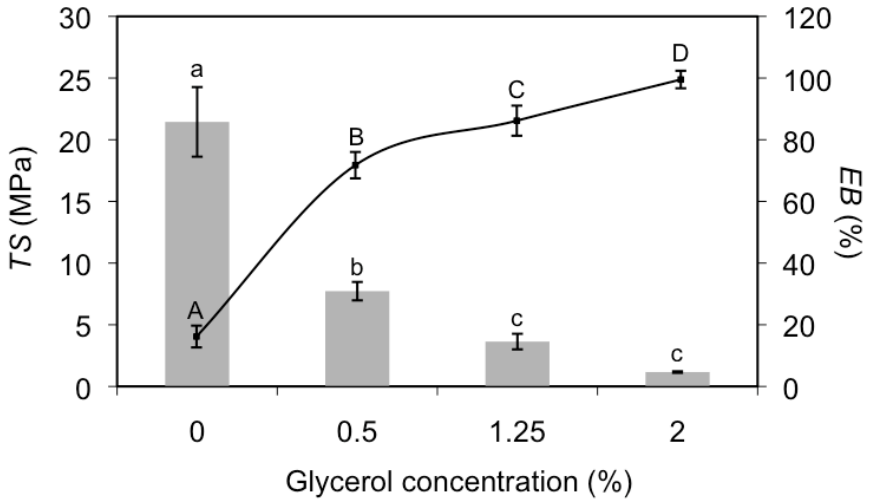

Figure 5-9. Tensile strength (*TS*) (bars) and elongation-at-break (*EB*) (line) of chitosan films for increasing glycerol concentrations. [a–d;A-D]Means with different superscripts are significantly different (p<0.05).

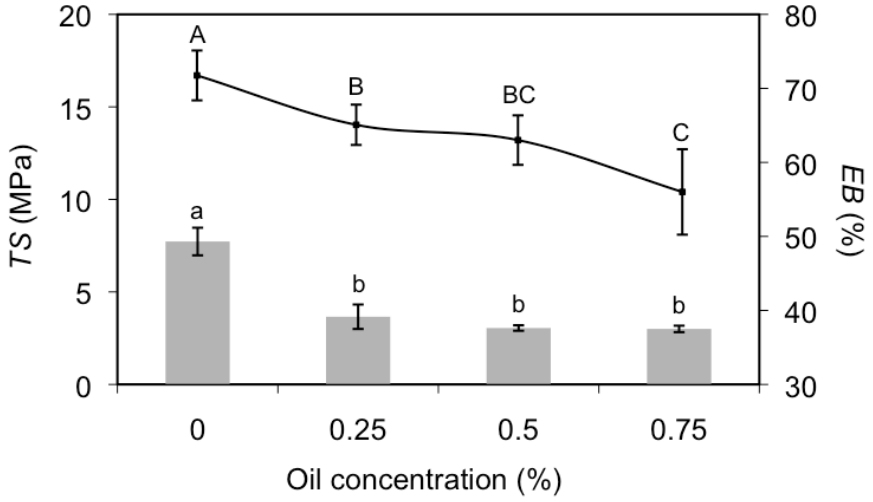

Figure 5-10. Tensile strength (*TS*) (bars) and elongation-at-break (*EB*) (line) of chitosan films for increasing oil concentrations. [a–d]Means with different superscripts are significantly different (p<0.05).

5.3.2.7 Opacity

The opacity means a smaller transparency, which is important e.g. to control the incidence of light on the food product; it is a relevant property since it has a direct impact on the appearance of the coated product. Figure 5-11 shows the influence of glycerol and oil concentrations in the opacity of chitosan films. The increase of glycerol concentration leads to a decrease of the opacity, possibly explained by the changes of the free volume of the polymer network, which lead to an increased mobility of the polymer chains and decrease the opacity by permitting a better penetration of the light. However, this behaviour only has a statistically significant difference (p<0.05) when a concentration of 0.5 % of glycerol is added to the films. The presence of oil increases the opacity values, however this difference is statistically significant (p<0.05) only for oil concentrations of 0.75 %. The increase of opacity when oil was added, is a direct consequence of the presence of lipid droplets dispersed in the chitosan film. These results are in agreement with other reported results, where lipid compounds increased the opacity of polysaccharide films (Yang & Paulson 2000; Park et al., 2004).

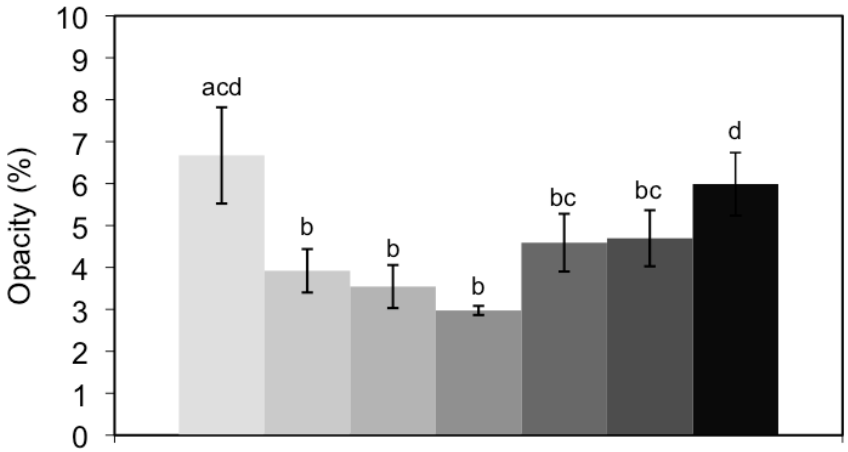

Figure 5-11. Opacity of chitosan (CH) films for increasing glycerol (Gly) and oil concentrations (1.5 % CH ; 1.5 % CH – 0.5 % Gly ; 1.5 % CH – 1.25 % Gly ; 1.5 % CH – 2.0 % Gly ; 1.5 % CH – 0.5 % Gly – 0.25 % Oil ; 1.5 % CH – 0.5 % Gly – 0.5 % Oil ; 1.5 % CH – 0.5 % Gly – 0.75 % Oil . [a–d]Means with different superscripts are significantly different (p<0.05).

5.3.3. Galactomannan film forming solutions

5.3.3.1 Wettability

The liquid-vapour interfacial tension (γ_{LV}) of galactomannan film forming solutions are presented in Table 5-5 and Table 5-6. Results show that the increase of glycerol and oil concentrations lead to a decrease of γ_{LV} for the galactomannan film forming solutions. As explained for chitosan film forming solutions, the increase of glycerol and oil concentrations lead to a higher surface pressure decreasing the liquid-vapour interface (Choi et al., 2002). The obtained values are in agreement with other reported values for film forming solutions of polysaccharides (Choi et al., 2002; Ribeiro et al., 2007; Ziani et al., 2008; Fabra et al., 2009;).

Table 5-5 and Table 5-6 show the W_s values of the film forming solutions on the reported surfaces as a function of increasing concentrations of glycerol and oil, respectively. The surface of SS316, that presents the lowest polar component, also displays the highest (p<0.05) W_s value when compared with the W_s values for the other surfaces. Moreover, when corn oil is added to the film forming solutions the W_s values increase, showing that the hydrophobic character of the oil has an important role in galactomannan film forming solutions, when interacting with surfaces. Table 5-6 shows that W_s values increased (p<0.05) when 0.5 % of oil was added to the galactomannan solutions. This behaviour was observed for all the solid surfaces under evaluation. The dispersive components of the solids had a great influence when oil was added to the film forming solutions. The glass and PMMA surfaces presented values of dispersive component closer to each other (p>0.05) while SS316 presented a lower value (Table 5-1), which can explain the differences observed in W_s values.

Figure 5-12 shows microscopy images of the film forming solutions of galactomannan. The presence of oil in the film forming solutions lead to the presence of oil particles (Figure 5-12(b, c and d)). The increase of oil concentrations lead to oil particles with a bigger size, ranging between 10 and 100 μm. Film forming solutions of galactomannan show an emulsifying capacity, that was already stated by other authors (Huang et al., 2001; Dickinson, 2003). It has been shown that galactomannans have the capacity to emulsify oil at a moderately low galactomannan/oil ratio (Dickinson, 2003); this behaviour might explain the lower numbers of

lipid particles observed by microscopy images, since a relatively high ratio galactomannan/oil was used in this film forming solutions.

Table 5-5. Values of liquid-vapour interfacial tension (γ_{LV}) and the spreading coefficient (W_s) of the film forming solutions of 1.5 % galactomannan for increasing glycerol concentrations

Glycerol % (w/v)	γ_{LV} (mN m^{-1})	W_s (mN m^{-1})		
		PMMA	Glass	SS316
0	50.38±2.87^{a}	-39.79±0.92$^{a;A}$	-37.32±1.11$^{a;A}$	-51.54±1.04$^{a;B}$
0.5	46.39±2.45^{b}	-32.92±0.71$^{b;A}$	-33.31±0.92$^{b;A}$	-36.37±0.81$^{b;B}$
1.25	46.03±1.75^{b}	-32.90±0.59$^{b;A}$	-34.18±1.06$^{b;A}$	-35.28±1.08$^{bc;B}$
2	39.03±3.29^{c}	-27.75±1.03$^{c;A}$	-31.17±1.36$^{c;B}$	-34.32±0.90$^{c;C}$

$^{a-c,A-C}$ Means in the same column with different superscripts (lower case) are significantly different (p<0.05). For the W_s values, means in the same line with different superscripts (upper case) are significantly different (p<0.05).

Table 5-6. Values of liquid-vapour interfacial tension (γ_{LV}) and the spreading coefficient (W_s) of the film forming solutions of 1.5 % galactomannan and 0.5 % of glycerol for increasing oil concentrations

Oil % (w/v)	γ_{LV} (mN m^{-1})	W_s (mN m^{-1})		
		PMMA	Glass	SS316
0	46.39±2.45^{a}	-32.92±0.71$^{a;A}$	-33.31±0.92$^{a;A}$	-36.37±0.81$^{a;B}$
0.25	38.57±3.53^{a}	-40.34±2.49$^{b;A}$	-37.31±2.26$^{b;A}$	-36.82±2.37$^{a;A}$
0.5	37.42±3.75^{a}	-46.13±3.53$^{c;A}$	-38.86±3.26$^{b;B}$	-39.15±3.00$^{b;B}$
0.75	35.19±1.79^{a}	-43.69±1.65$^{b;A}$	-40.83±1.48$^{c;AB}$	-40.22±0.87$^{b;B}$

$^{a-c,A-C}$ Means in the same column with different superscripts (lower case) are significantly different (p<0.05). For the W_s values, means in the same line with different superscripts (upper case) are significantly different (p<0.05).

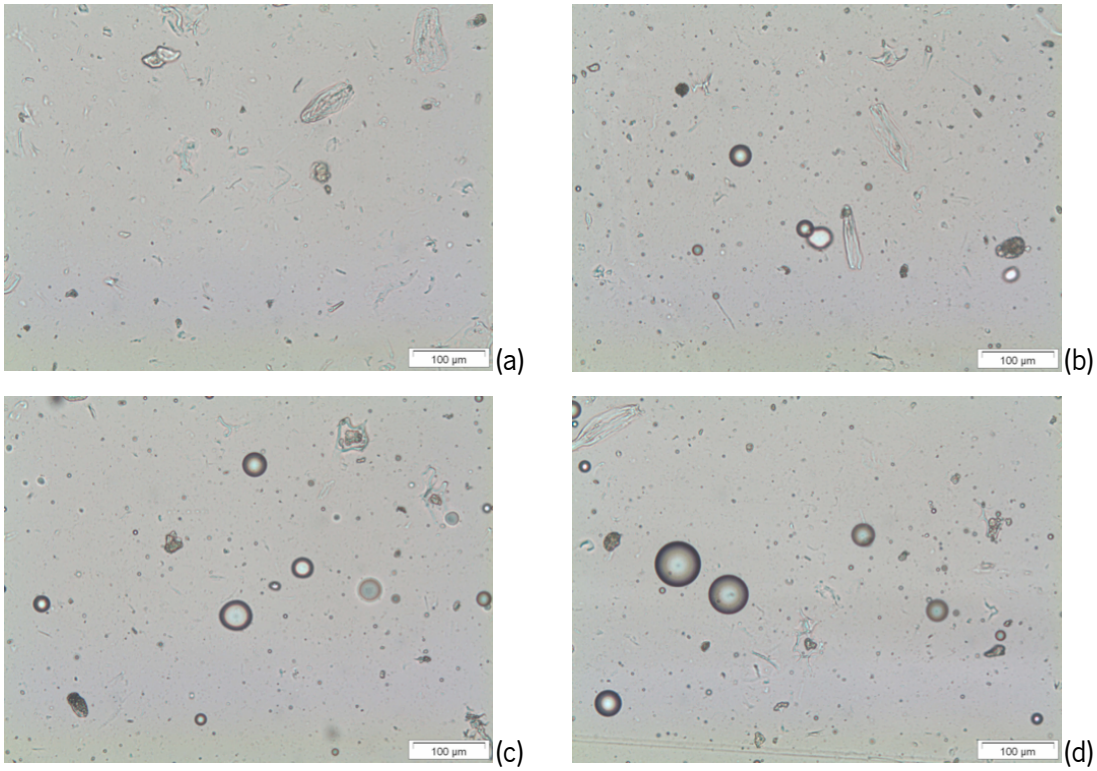

Figure 5-12. Optical microscopy images (100 ×) of galactomannan film forming solutions for: 1.5 % of galactomannan with 0.5 % of glycerol: without oil (a); 0.25 % of oil (b); 0.5 % of oil (c) and 0.75 % of oil (d).

5.3.3.2 Fourier-transform infrared (FTIR) and moisture content

Figure 5-13 shows the FTIR spectra of galactomannan films for increasing glycerol concentrations. The broad band ranging between 3500 and 3100 cm^{-1} is attributed to O-H stretching vibration formed by the hydroxyl group of galactomannan and water (Yuen et al., 2009). For higher glycerol concentrations the intensity of the band increases, due to the hydrogen bonds formed by the hydroxyl groups of both galactomannan and glycerol. This behaviour is supported by the values of moisture content of the film for increasing glycerol concentrations (Figure. 5-14).

The broad band around 2800-3000 cm^{-1} is attributed to C-H stretch. The bands 1148 and 1092 cm^{-1} result from the stretching vibration of C-O in C-O-H bonds, however the band at 1092 cm^{-1} only appears for galactomannan films containing 1.25 % and 2.0 % of glycerol. The

broad band at 1016 cm^{-1} that corresponds to the stretching vibration of C-O in C-O-C bonds appears in all galactomannan film samples. However, the broad band at 925 cm^{-1} that also corresponds to the stretching vibration of C-O in C-O-C bonds only appears in the films containing glycerol, confirming the higher amounts of C-O bonds in the films' structure (Jamróz et al., 2007; Pelissari et al., 2009).

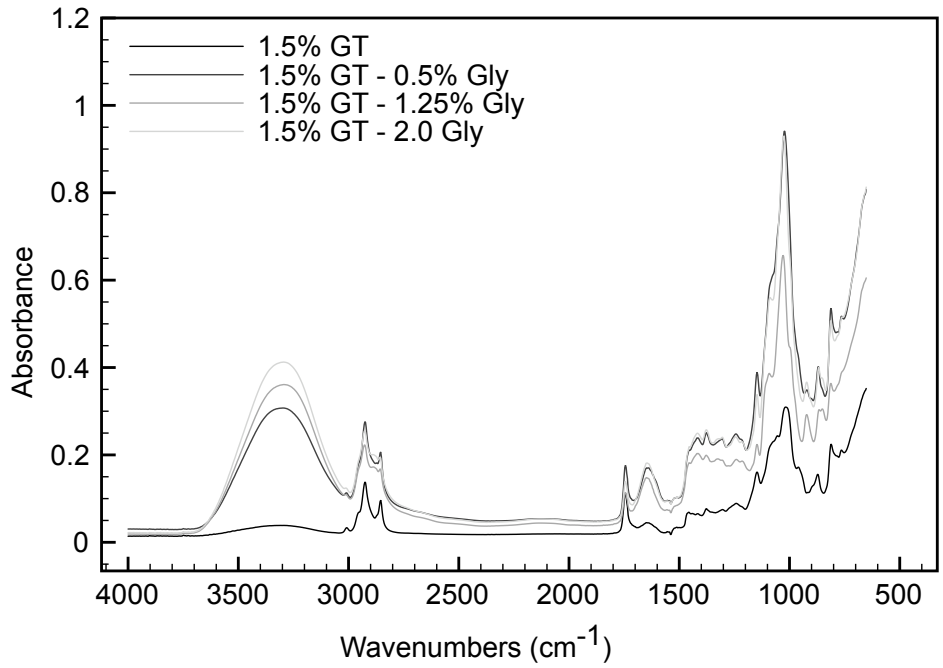

Figure 5-13. FTIR spectra of the galactomannan (GT) films for increasing glycerol (Gly) concentrations.

Figure 5-14 and Figure 5-15 show the variations of the moisture content in galactomannan films for increasing concentrations of glycerol and oil, respectively. Water is not only associated with the galactomannan film's structure, but also with the glycerol hydrophilic nature that retains water in the matrix. Higher concentrations of plasticizer favour the adsorption of water molecules, which is mainly attributed to the predisposition of plasticizers to form hydrogen bonds.

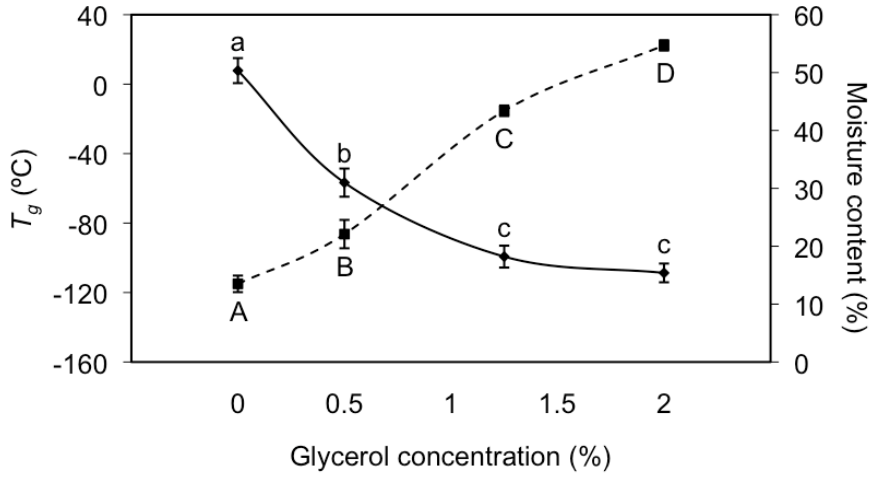

Figure 5-14. Glass transition temperatures (T_g) (—) and moisture content (- - -) of galactomannan films for increasing glycerol concentrations. $^{a-c;A-D}$Means with different superscripts are significantly different (p<0.05).

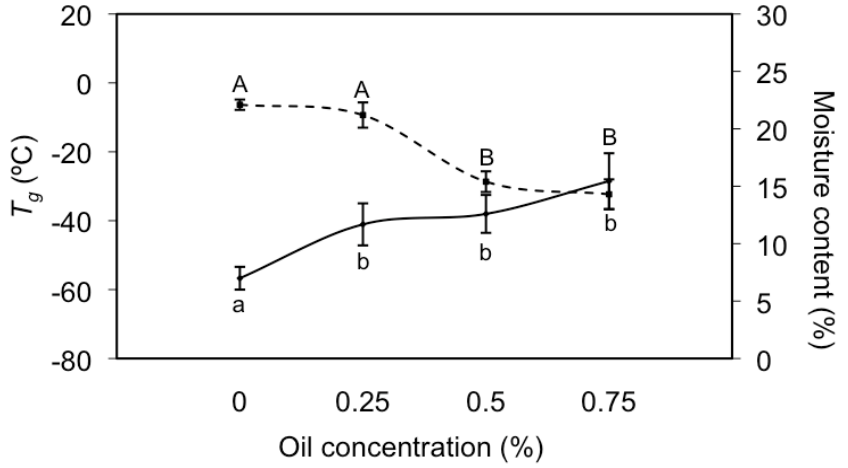

Figure 5-15. Glass transition temperature (T_g) (—) and moisture content (- - -) of galactomannan films for increasing oil concentrations. $^{a-b;A-B}$Means with different superscripts are significantly different (p<0.05).

Figure 5-16 shows FTIR spectra of galactomannan films for increasing oil concentrations. A decrease in the water content is observed for galactomannan films without and with oil, sequentially. Galactomannan films with oil show more intense peaks in the frequency range between 2800 and 3100 cm^{-1} that can be related with the presence of oil. A band at approximately 3009 cm^{-1} was associated with the C-H stretching vibration of the *cis*-double bond of the fatty acids presente in corn oil (Vlachos et al., 2006). Also the peak at 1457 cm^{-1} observed in films with oil can be related with the bending vibrations of the aliphatic groups (CH_3 and CH_2) (Vlachos et al., 2006). The broad band ranged between 3500 and 3100 cm^{-1}, associated to the O-H stretching vibration, shows a intensity decrease associated with hydroxyl bonds of the films that decrease in number when oil is added to the matrix (Yuen et al., 2009).

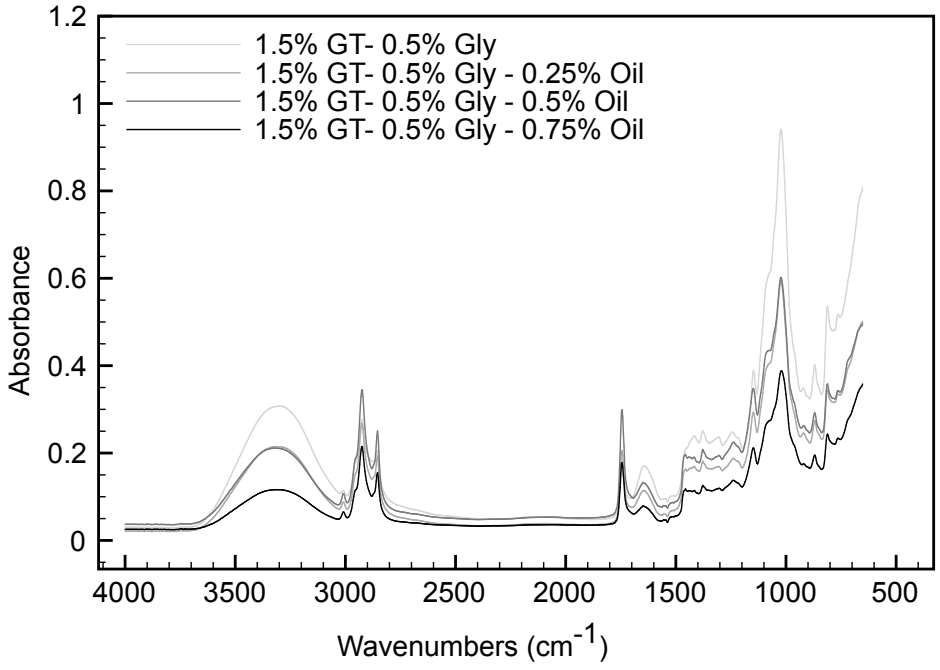

Figure 5-16. FTIR spectra of the galactomannan (GT) films for increasing oil concentrations.

5.3.3.3 Thermal analyses

Figure 5-14 shows T_g of galactomannan films for different glycerol concentrations. Results show that the glycerol presence decreases (p<0.05) the values of T_g of galactomannan films. Higher glycerol concentrations lead to the increase of the free volume and the mobility of molecules changing the physical structure of the film, and decreasing the T_g of polysaccharides (Roos & Karel, 1991). Moreover, T_g values are inversely associated with the water content of the galactomannan films; also, the water acts as a plasticizer increasing the molecular mobility (lower T_g values) of the galactomannan films. The presence of glycerol leads to the presence of more hydrogen bonds in the film matrix, as confirmed by FTIR analyses (Figure 5-13). The T_g values obtained for galactomannan films are in agreement with reported results for galactomannan films from other sources (Mikkonen et al., 2007).

Oil incorporation decreases the mobility of the galactomannan matrix (increase of T_g values) that can be related with the oil structure, but can also be influenced by the decrease of the moisture content of films (Figure 5-15).

Figure 5-17 shows the variation of ΔH_m and T_m of galactomannan films with the increase of glycerol concentrations. For films without glycerol and for films with oil concentrations above 0.5 % the melting peak was not observed. The thermograms (results not shown) had a flat shape indicating an amorphous structure of the films (Mathew et al., 2002; Yakimets et al., 2007). Results show that the increase of glycerol concentrations lead to higher values of ΔH_m, explained by the improved crystallinity of the film (Souza et al., 2010). When glycerol concentration increased a greater polymer mobility (lower T_g values) is achieved that favours the formation of crystalline domains, leading to more crystalline films with higher ΔH_m values (Fabra et al., 2010; Mathew et al., 2002). Also, the increase of the moisture content in the film matrix when more hydrogen bonds are available can influence the crystallinity of the films (Chen et al., 2008). T_m values were not influenced (with statistical significance, p>0.05) by the increase of glycerol concentration.

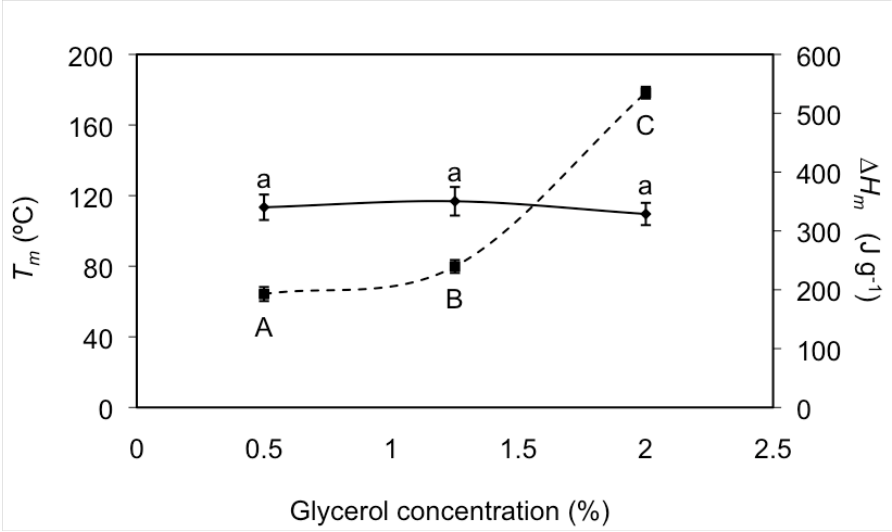

Figure 5-17. Melting temperature peak (T_m) (—) and enthalpy of melting (ΔH_m) (- - -) of galactomannan films for increasing glycerol concentrations. $^{a-b;A-C}$Means with different superscripts are significantly different (p<0.05).

Table 5-7 shows the peak values of thermal events and the corresponding weight loss associated with increasing temperature in galactomannan films. Thermal analyses show that galactomannan films without glycerol are stable up to 40 °C, and when glycerol is added this stability increases for 59 °C (results not shown). Galactomannan films show at least three thermal events, the first being attributed to water evaporation, the second (around 200 °C) attributed to the presence of glycerol, and the third related to polysaccharide decomposition (Zohuriaan & Shokrolahi, 2004). For samples containing oil a fourth event was observed related with the aromatic structures present in corn oil with decomposition temperatures above 380 °C (Pelissari et al., 2009). Peak events show that the increase of glycerol concentration leads to a more significant weight loss associated with two thermal events: water evaporation (peak 1), and chemisorbed water through the hydrogen bonds favoured by the glycerol presence (peak 2) (Quijada-Garrido et al., 2007). In films with oil a weight loss associated with oil decomposition is observed (peak 4), in which for higher corn oil concentrations the weight loss increases.

Table 5-7. Thermal behaviour of galactomannan films. The values of the peaks correspond to the values of derivative thermograms obtained from the TGA curve

Films* 1.5 % GT	Peak 1 (°C)	Weight loss (%)	Peak (°C)	Weight loss (%)	Peak 3 (°C)	Weight loss (%)	Peak 4 (°C)	Weight loss (%)
-	47.1	-7.7	-	-	301.6	-40.2	-	-
0.5 % Gly	59.3	-7.3	188.8	-10.6	306.3	-34.6	-	-
1.25 % Gly	68.3	-14.3	207.6	-32.0	309.1	-27.5	-	-
2.0 % Gly	80.6	-16.1	217.9	-51.4	310.1	-14.5	-	-
0.5 % Gly-0.25 % Oil	70.2	-9.6	247.0	-10.1	307.8	-34.6	421.8	-6.0
0.5 % Gly-0.5 % Oil	75.2	-8.7	234.3	-9.9	309.4	-33.8	403.5	-13.9
0.5 % Gly-0.75 % Oil	60.1	-7.2	248.0	-8.3	309.4	-32.5	417.6	-17.5

*Films with 1.5 % of galactomannan (GT) and increasing concentrations of glycerol (Gly) and oil.

5.3.3.4. Water solubility

Figure 5-18 shows the solubility values of galactomannan films. Results indicate that both glycerol and oil influence the water solubility of galactomannan films. The presence of oil and the increase of glycerol concentration show different effects. The increase of glycerol concentration leads to an increase (p<0.05) of the solubility. The increase of the hydrophilicity of the film matrix with the increase of glycerol concentration leads to a higher solubility of the films. FTIR studies showed an increase of O-H bonds with the increase of glycerol content, leading to a greater interaction of the film matrix with water. Also the mobility of the matrix (lower T_g) has a great influence in these results. In a system with more mobility, water molecules enter more easily in the film matrix thus increasing its solubility.

Oil addition (from 0 to 0.25 %) promotes a slight increase (p>0.05) of solubility. However, for oil concentrations of 0.75 % a significant decrease of the solubility (p<0.05) occurs. The hydrophobic character of oil changes the film structure leading to a less soluble film. The presence of oil decreases the number of O-H bonds in the galactomannan film. Also, the presence of an aliphatic group in galactomannan films when oil is added can lead to a decrease of solubility values (Morillon et al., 2002).

The results obtained are in agreement with the solubility values for other polysaccharide based films (Mehyar & Han, 2004; Casariego et al. 2009; Piermaria et al., 2009).

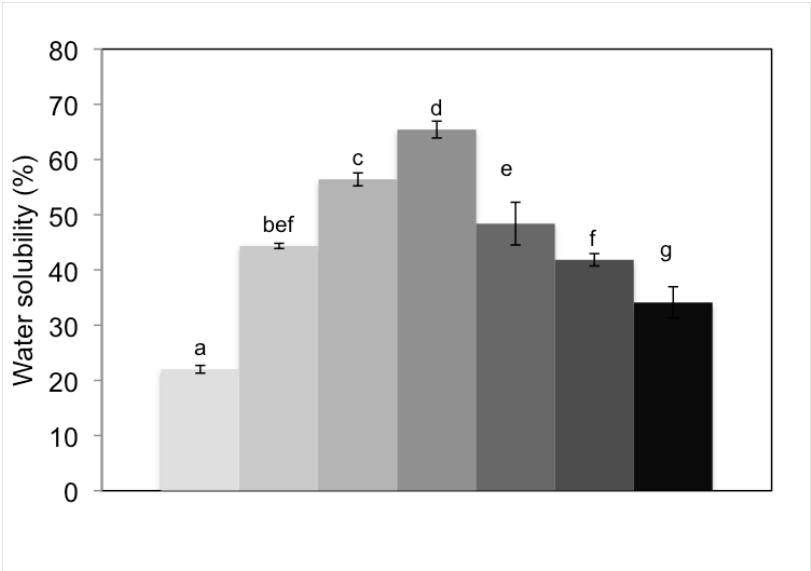

Figure 5-18. Water solubility of galactomannan (GT) films for increasing glycerol (Gly) and oil concentrations (1.5 % GT ; 1.5 % GT – 0.5 % Gly ; 1.5 % GT – 1.25 % Gly ; 1.5 % GT – 2.0 % Gly ; 1.5 % GT – 0.5 % Gly – 0.25 % Oil ; 1.5 % GT – 0.5 % Gly – 0.5 % Oil ; 1.5 % GT – 0.5 % Gly – 0.75 % Oil . [a-g]Means with different superscripts are significantly different (p<0.05).

5.3.3.5. Water vapour permeability (*WVP*)

Figure 5-19 shows the influence of glycerol and oil in the *WVP* of galactomannan films. When 0.5 % of glycerol is added to the galactomannan film a slight decrease (p>0.05) of *WVP* values is

observed. However, when glycerol concentration increases from 0.5 % to 1.25 % and then to 2.0 % the *WVP* values increase significantly. Glycerol and its plasticizing effect increase the molecular mobility and decrease the rigidity of polysaccharide chains. In the first case, the presence of the plasticizer decreases the occurrence of cracks and pores, improving the dispersion and flexibility of the film and thus decreasing gas permeability (Garcia et al., 2000). In the second case, the higher concentrations of plasticizer favour molecular mobility (decreasing T_g values) and the adsorption of water molecules, increasing *WVP* values (Diab et al., 2001).

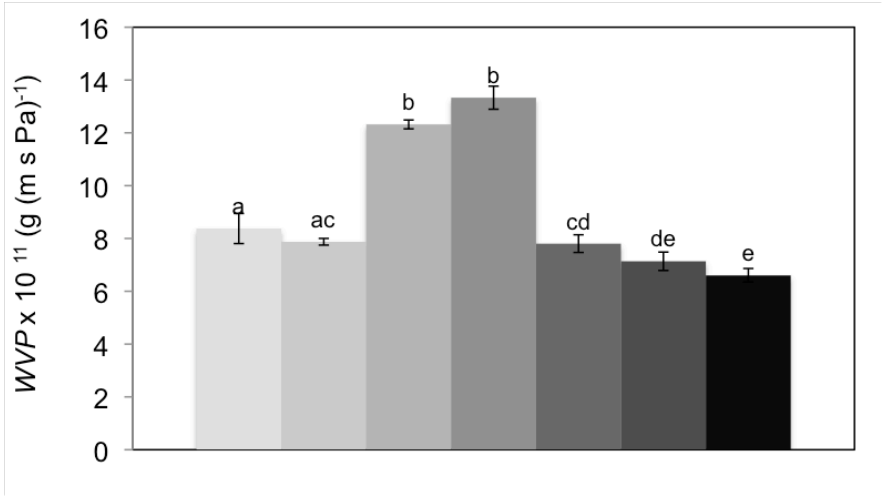

Figure 5-19. Water vapour permeability (*WVP*) of galactomannan (GT) films for increasing glycerol (Gly) and oil concentrations (1.5 % GT ; 1.5 % GT – 0.5 % Gly ; 1.5 % GT – 1.25 % Gly ; 1.5 % GT – 2.0 % Gly ; 1.5 % GT – 0.5 % Gly – 0.25 % Oil ; 1.5 % GT – 0.5 % Gly – 0.5 % Oil ; 1.5 % GT – 0.5 % Gly – 0.75 % Oil . $^{a-e}$Means with different superscripts are significantly different (p<0.05).

Figure 5-19 shows that the increase of oil concentration leads to lower *WVP* values, however these differences are statistically significant (p<0.05) only for oil concentrations higher than 0.25 %. The hydrophobic character of the oil blended with galactomannan polysaccharide changes the film properties decreasing their *WVP*. The decrease of the *WVP* of the films with the addition of oil can be justified by the reduction of the hydrophilic portion of the film and by the presence of the aliphatic group (Morillon et al., 2002; Hernandez-Munõz et al., 2004), that leads to a more hydrophobic film, thus decreasing the water affinity of the film and consequently

decreasing *WVP*. In the present work, the lowest values of *WVP* were obtained for films made with a mixture of 1.5 % of galactomannan, 0.5 % glycerol and with 0.75 % of oil. These values are in agreement with those obtained in other works with galactomannan films (Aydinli & Tutas, 2000).

5.3.3.6. Tensile strength (*TS*) and elongation-at-break (*EB*)

Figure 5-20 shows how glycerol concentrations affect *TS* of galactomannan films. The galactomannan films without glycerol present a typical behaviour of an unplasticized film: high values of *TS* and a great variability due to their unhomogeneous structure. It is important to note that galactomannan films without glycerol at room temperature are in the glass-rubber transition zone (Figure 5-14), which from a molecular point of view involves molecular motion and the beginning of reptation (Sperling, 2006), leading to a non-uniform structure (Roos & Karel, 1991). The increase of glycerol concentration (from 0.5 % to 2.0 %) had a statistically significant influence ($p<0.05$) on the values of *TS* and *EB*. This increase lead to *TS* values 87 % lower and values of *EB* 3.2-fold higher than those found in films without glycerol. As previously mentioned, plasticizers interfere with galactomannan chains where, by decreasing intermolecular forces, they reduce the rigidity of the film structure and increase the polymer mobility, thus facilitating film elongation, in accordance with the T_g values obtained in the present work.

Figure 5-21 shows that the presence of oil influences both *TS* and *EB* values for the galactomannan films. The oil incorporation leads to a decrease of *TS* and an increase of *EB* values when compared with the film without oil. This decrease only presents statistical significance ($p<0.05$) for oil concentrations above 0.25 %. The results seem to indicate that oil acts as a plasticizer leading to a less rigid film structure, increasing its flexibility and decreasing its resistance to fracture. This behaviour can also be related with the *cis*-double bonds, present in films containing oil, that possibly contribute to increase their flexibility (Morillon et al., 2002). In the present work, the highest values of *TS* were obtained for the formulation containing 1.5 % of galactomannan (18.6 MPa); and the highest value of *EB* was obtained for the film formulation containing 1.5 % of galactomannan and 2.0 % of glycerol (38.7 %). These results are in agreement with other reported results for galactomannan films (Mikkonen et al., 2007; Martins et al., 2010).

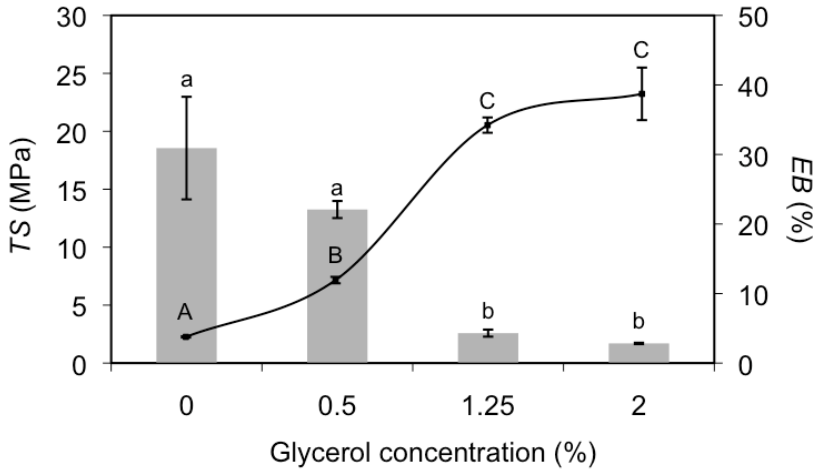

Figure 5-20. Tensile strength (*TS*) (bars) and elongation at break (*EB*) (line) of galactomannan films for increasing glycerol concentrations. [a–b;A-C]Means with different superscripts are significantly different (p<0.05).

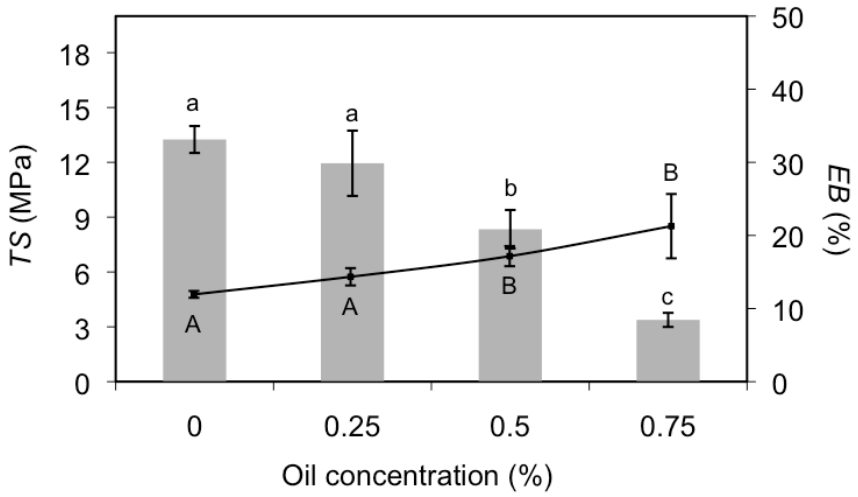

Figure 5-21. Tensile strength (*TS*) (bars) and elongation at break (*EB*) (line) of galactomannan films for increasing oil concentrations. [a–c;A-B]Means with different superscripts are significantly different (p<0.05).

5.3.3.7 Opacity

Figure 5-22 shows that glycerol and oil concentrations have a statistically significant influence (p<0.05) in the galactomannan film opacity. The increase of glycerol concentration leads to an increase of the free volume of the polymer network, decreasing the opacity by permitting a more efficient penetration of light. Also, the presence of oil has a statistically significant influence (p<0.05) on opacity, leading to more opaque films. The increase of opacity when oil is added was probably a result from the presence of lipid droplets formed during the coating formulation that are dispersed in the polymer matrix immediately after the film is formed. These results are in agreement with reported results from (Yang & Paulson, 2000; Park et al., 2004).

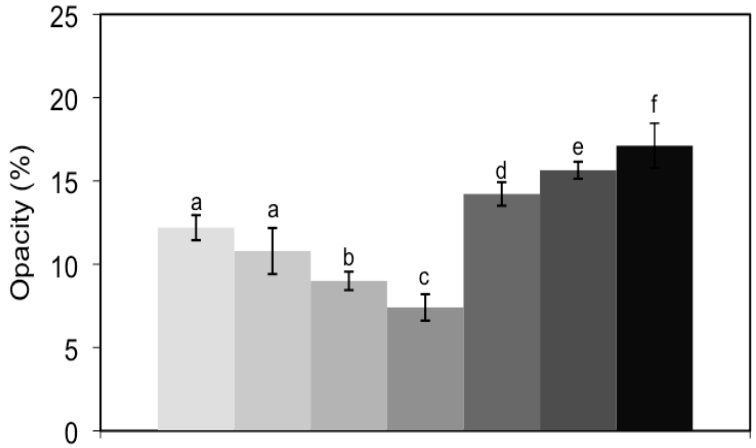

Figure 5-22. Opacity values of galactomannan (GT) films for increasing glycerol (Gly) and oil concentrations (1.5 % GT ; 1.5 % GT – 0.5 % Gly ; 1.5 % GT – 1.25 % Gly ; 1.5 % GT – 2.0 % Gly ; 1.5 % GT – 0.5 % Gly – 0.25 % Oil ; 1.5 % GT – 0.5 % Gly – 0.5 % Oil ; 1.5 % GT – 0.5 % Gly – 0.75 % Oil . $^{a-f}$Means with different superscripts are significantly different (p<0.05).

5.3.4 Comparison between properties of chitosan and galactomannan films

The presence of glycerol and oil were shown to have different influences on film forming solutions, depending on the polysaccharide used and on the tested surface. When glycerol is added, the liquid-vapour interfacial tension decreases for both film forming solutions (chitosan and galactomannan) leading to a decrease of the spreading coefficient (W_s) while when oil is added this behaviour was not observed. The values of W_s of galactomannan film forming solutions increase when oil is added, while for chitosan film forming solutions the values of W_s decrease for all the surfaces. This distinct behaviour between chitosan and galactomannan film forming solutions can be explained by the emulsifying properties of each film forming solutions. Chitosan, with the utilization of Tween 80, forms micelles where the oil droplets can be encapsulated. On the other hand, galactomannan film forming solutions, despite having a moderate emulsifying capacity, are not able to emulsify all the oil, which will be more available to interact with the surface of the solids, increasing the values of W_s. The tested surfaces present similar dispersive components and have a more pronounced difference in the polar component. Chitosan film forming solutions shows more affinity to the surface with the higher polar component (PMMA), and the same happens for the galactomannan film forming solutions without oil. For galactomannan film forming solutions with oil the surfaces with the lower polar component (SS316 and glass) are the ones showing the lowest W_s values.

The study of the physicochemical properties of polysaccharide films show how the presence of glycerol and/or corn oil can influence their structural reorganization. Water adsorption on the films' matrix for different glycerol and corn oil concentrations varies for both chitosan and galactomannan films, as shown by FTIR spectra and moisture content results. The major differences between the used polysaccharides, from a structural point of view, lay in the substitution of the O-H group by an N-H function in the case of chitosan. This means that glycerol and oil will influence the films' structure in a different way. While for the galactomannan the specific sorption sites for water are the O-H groups, for chitosan those are O-H and/or NH_2 groups (Despond et al., 2005), thus changing their capacity to interact with water.

Chitosan and galactomannan films present a similar behaviour when glycerol is added to the film. Glycerol leads to an increase of the moisture content; however, the obtained values for

chitosan films are higher and statistically different (p<0.05) from the values for galactomannan films. For films without glycerol moisture content values are 15.5 and 13.5 % for chitosan and galactomannan, respectively; however, when glycerol is added to the film forming solutions, the resulting chitosan films present a moisture content at least 12 % higher than galactomannan films. This behaviour can be explained by the presence of Tween 80 in all chitosan films. Tween 80 presents a hydrophilic-lipophilic balance (HLB) of 15 that indicates that the surfactant is readily soluble in water (Carneiro-da-Cunha et al., 2009), increasing the ability of the chitosan matrix to adsorb water molecules. The water sorption occurs in two main steps: the water sorption on polymer sites and the water clustering surrounding the firstly sorbed water molecules (Fringant et al., 1996). The plasticizing effect of glycerol leads to a film matrix with more mobile regions with larger interchain distances, thus allowing the sorption of more water molecules to polysaccharides sites. Besides, the glycerol structure creates more hydroxyl bonds that promote the sorption of water molecules to the film.

Both polysaccharides are strongly water content dependent, however they present different behaviours when their thermal, transport and mechanical properties are compared. The major differences are observed in the glass transition temperature (T_g), water vapour permeability (*WVP*) and elongation-at-break (*EB*) values. T_g values for the two polysaccharide films show the well-known plasticizing effect of glycerol, which results in a decrease of T_g. However, they are in different states at ambient temperature. While chitosan films are in the glassy state, with T_g values higher than 20 °C (with exception of chitosan films containing 2.0 % of glycerol), the galactomannan films are in the rubbery state (with exception of galactomannan films without glycerol, that present a brittle behaviour; this means that glycerol is necessary for a correct processing of these films). Galactomannan films without glycerol, at room temperature, are in the glass-rubber transition zone leading to a non-uniform structure (Roos & Karel, 1991). *TS* values of galactomannan films without glycerol show an unplasticized film behaviour with a great variability due to their unhomogeneous structure. However, for films containing glycerol (0.5 %) it can be observed that *TS* values for galactomannan films are higher than those obtained for chitosan films. This behaviour can be justified by the linear and neutral nature of the polymer chains of the galactomannan films, which can associate more intimately through intermolecular hydrogen bonding (Nieto, 2009). Chitosan films present higher values for *EB* than

galactomannan films, being this difference more evident for higher glycerol concentrations. The chitosan structure is more flexible due to the presence of N-H bonds and this may be the reason for the observed behaviour. Also, when oil is added, chitosan and galactomannan films present distinct behaviour for *EB* values. While for chitosan the presence of oil leads to lower values of *EB*, for galactomannan films this presence increases the *EB values.* This behaviour can be related with the emulsifying capacity of the chitosan film forming solutions, higher than that of galactomannan film forming solutions; when the films are cast, oil increases the structural discontinuities decreasing film flexibility.

5.4 CONCLUSION

The presence of glycerol and corn oil leads to changes in the polysaccharide coatings/films structure, with the formation of new and/or the increase of the number of existing bonds in both the film forming solution and film structure. These bonds influence the water affinity of the films, and consequently change their properties. It has been shown how coatings/films are influenced by the presence of plasticizer and/or oil. The presence of these components influences coatings/films' physicochemical properties as determined by the liquid-vapour interfacial tension of the film forming solutions, and by their wettability on three different surfaces. Also the films' structure is influenced by the presence of glycerol and oil, as confirmed by the results for transport, thermal and mechanical properties, solubility and opacity.

The chitosan and galactomannan films evaluated in this work provide water vapour permeability and elongation-at-break values in the range of e.g. cellophane films; they also show in some cases tensile strength values close to those reported for high-density polyethylene and low-density polyethylene (Han et al., 2005), which indicate that they can be viable alternatives to synthetic packaging materials.

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CHAPTER 6

EVALUATION OF POLYSACCHARIDES AS EDIBLE COATINGS FOR CHEESE

The main objective of this chapter was to study the ability of chitosan and galactomannan solutions to be used as coatings for cheese. Chitosan and galactomannan coatings/films, with different formulations, were evaluated based on their properties. The better solutions for each polysaccharide were chosen based on their wettability on cheese, and then on their transport properties, opacity and mechanical properties as well.

6.1 INTRODUCTION

In the last years, the food and packaging industries joined efforts to reduce the amount of food packaging materials, once the environmental issues became important to the consumer. As an answer to that concern, several problems were addressed in order to foster the commercial use of bio-based primary food packaging materials. These problems include degradation rates under various conditions, changes in mechanical properties during storage, potential for microbial growth, and release of harmful compounds into the packaged food product (Petersen et al., 1999; Siracusa et al., 2008). On the other hand, consumers around the world demand for food of high quality, without chemical preservatives, and with an extended shelf-life. Therefore, an increased effort has been made to discover new natural preservatives (Petersen et al., 1999).

The use of edible coatings creates a modified atmosphere surrounding the commodity similar to that achieved by controlled or modified atmospheric storage conditions. The modified atmosphere created by edible coatings can protect the food from the moment it is applied, through transportation to its final retail destination, and in the home of the consumer (Kester & Fennema, 1986; Petersen et al., 1999).

The application of the coating can be achieved by: dipping the product into, or by brushing or spraying it with the coating solution. Depending on the formulation of the coating solution, the product will absorb an appropriate amount of coating material necessary to form the desired layer, which when dried, protects the food surface (Pavlath et al., 2009). The wettabilty of film forming solution materials is an important parameter for the coating process. A deficient wettability of the film forming solution on the food surface can lead to an incomplete coating or easy peel-off of the coating layers from the surface (Han et al., 2005).

The present work evaluates the possibility of using polysaccharides (chitosan and galactomannan) as coatings applied on semi-hard cheese. The choice of the best coating is made taking into consideration its wettability, transport properties, opacity and mechanical properties.

6.2 MATERIALS AND METHODS

6.2.1 Film forming solutions and films preparation

Chitosan film forming solutions were prepared dissolving chitosan (0.5 and 1.5 % w/v) in a lactic acid (1.0 % v/v) solution with the addition of Tween 80 (0.2 %) under agitation using a magnetic stirrer (at 200 rpm) overnight at room temperature (20 °C). Galactomannan film forming solutions were prepared dissolving the lyophilized galactomannan (0.5 and 1.5 % w/v) in distilled water, using a magnetic stirrer (at 200 rpm) overnight at room temperature (20 °C). Corn oil (Sovena, Portugal) was added to the film forming solutions in concentrations of 0.5 % (w/v) under agitation during 20 min at 60 °C. Glycerol (87 %, Panreac, Spain) was added in two different concentrations (0.5 and 2.0 % w/v).

Glycerol and oil concentrations were chosen based on the results obtained in Chapter 5, where the most significant differences were observed for values of 0.5 and 2.0 % of glycerol and 0 and 0.5 % of corn oil.

To produce the films, a constant amount (13 mL) of film forming solution was cast onto a 5.7 cm diameter Petri plate. The films were dried in an oven at 35 °C during 16 h. Films were maintained at 23 °C and 54 % RH at least 24 h before performing the tests (these conditions were obtained in a dessicator through a saturated salt solution of $Mg(NO_3)_2$ which was periodically replaced).

6.2.2 Cheese preparation for contact angle measurements

Before testing, the cheese was left at room temperature (20 °C) until a uniform temperature was achieved in its whole volume and the surface was cleaned with distilled water. Cheese skin portions with 1 cm of thickness were cut with a knife and placed on a glass plate for contact angle measurements.

6.2.3 Critical surface tension and wettability

According to Zisman (1964), in systems having a surface tension lower than 100 $mN \cdot m^{-1}$ (low-energy surfaces), the contact angle formed by a drop of liquid on a solid surface will be a linear function of the surface tension of the liquid, γ_{LV}, (where phase *V* is air saturated with the vapour of the liquid, *L*). The Zisman method is applicable only for low energy surfaces; it is therefore necessary to determine the surface energy of the cheese (Zisman, 1964). The surface tension measurements of the cheese were performed as explained in Chapter 5 (section 5.2.2.1).

The wettability of the coating solutions on cheese was studied by determining the values of the spreading coefficient (W_s) and the works of adhesion (W_a) and cohesion (W_c). The surface tension of the coating solution was measured by the pendant drop method using the Laplace-Young approximation (Song et al., 1996). The spreading coefficient was performed as explained in Chapter 5 (section 5.2.2.2). Measurements were made in less than 30 s. Twenty replicates of contact angle and surface tension measurements were obtained for each data point at 21.3 ± 0.5 °C.

6.2.4 Oxygen and carbon dioxide permeability

Oxygen permeability (O_2P) and carbon dioxide permeability (CO_2P) were determined based on the ASTM method (ASTM-D-3985-02, 2002). The films were sealed between two chambers, having each one two channels. In the lower chamber O_2 (or CO_2) was supplied at a controlled flow rate (J & W Scientific, ADM 2000, USA) to keep its pressure constant in that compartment. The upper chamber was purged by a stream of nitrogen, also at controlled flow. Nitrogen acted as a carrier for the O_2 (or the CO_2) crossing the film.

In the case of O_2P measurement, the flow leaving this chamber was connected to an O_2 sensor that measured the O_2 concentration in that flow on-line. In the case of CO_2P measurement the flow leaving this chamber was collected with a syringe for CO_2 quantification. To determine CO_2 concentration, 1 mL of sample (from the syringe) was injected in a gas chromatograph (Chrompack 9001, Middelburg, The Netherlands) at 110 °C with a column Porapak Q 80/100

mesh 2 m × 1/8" × 2 mm SS, using a thermal conductivity detector (TCD) at 110 °C. Helium at 23 mL min^{-1} was used as carrier gas.

A standard mixture containing 10 % of CO_2, 20 % of O_2 and 70 % of N_2 was used for calibration. The flows of the two chambers were connected to a manometer to ensure the equality of pressures (both at 1 atm) between both compartments. As the O_2 (and the CO_2) was carried continuously by the nitrogen flow, it was considered that O_2 (and CO_2) partial pressure in the upper compartment is null, therefore ΔP is equal to 1 atm. Three replicates were obtained for each sample, in each case (O_2P and CO_2P).

*6.2.5 Film thickness, water vapour permeability (*WVP*), tensile strength (*TS*), elongation-at-break (*EB*) and opacity*

Film thickness, *WVP*, and opacity measurements were performed as described in Chapter 4 (sections 4.2.7, 4.28 and 4.2.9, respectively); *TS* and *EB* were performed as described in Chapter 5 (section 5.2.10).

6.2.6 Statistical analysis

6.2.9.1. Design of experiments

Table 6-1 shows the settings (levels) used for each variable in the experiments. In the two sets (experimental setups I and II) a factorial design of 2^3 levels was applied, in a total of 8 non-centre-points runs. The independent variables were polysaccharide (chitosan and galactomannan), glycerol and oil concentrations in the experimental setup I and polysaccharide, glycerol/sorbitol mixture and oil concentrations in the experimental setup II.

Table 6-1. Factors and levels used to analyse polysaccharide coatings/films properties for experimental setup I and II

	Factors	Levels	
Experimental setup I	Polysaccharide	0.5 (-1)	1.5 (+1)
	Glycerol	0.5 (-1)	2.0 (+1)
	Oil	0.0 (-1)	0.5 (+1)
Experimental setup II	Polysaccharide	0.5 (-1)	1.5 (+1)
	Glycerol/Sorbitol	0.5 (-1)	2.0 (+1)
	Oil	0.0 (-1)	0.5 (+1)

6.2.9.2 Data analyses

Data analyses were performed using Microsoft Windows Excel 2003 and Statistica software (release 7, edition 2004, Statsoft, Tulsa, OK, USA). Pareto charts were drawn to express visually the statistical significance of each factor and the interactions between factors. The interaction plots show the means, where the means are indicated by points connected by lines, being useful to detect significant interaction effects in the evaluated parameters.

6.2.9.3 Modelling

The experimental data were fitted to a multifactor model, represented by Equation 6-1:

$$Y = a + b \cdot X_1 + c \cdot X_2 + d \cdot X_3 + e \cdot X_1 \cdot X_2 + f \cdot X_1 \cdot X_3 + g \cdot X_2 \cdot X_3 \quad \text{Eq. 6-1}$$

where Y_i represents the dependent variables: W_s, *WVP*, O_2P, CO_2P, Opacity, *TS* or *EB*, and X_n are the independent variables: polysaccharide (n = 1), glycerol (n = 2), and oil (n = 3) in the

experimental setup I; being the dependent variables represented in experimental setup II by Y_{ii} where the independent variables are: polysaccharide (n = 1), glycerol/sorbitol (n = 2), and oil (n = 3).

The fitting accuracy of the models was evaluated by the determination of the following parameters: coefficient of determination (R^2), mean relative deviation modulus (E) and accuracy factor (A_f). The meaning of the parameters R^2 and A_f are explained before in Chapter 4 (section 4.2.11).

The mean relative percentage deviation modulus, E, in Equation 6-2, indicates the goodness of the fit between the observed and predicted values of the analysed parameters for the independent variables used, being N the number of data points, R_{obs} the observed values of each parameter, and R_{pre} the values predicted by the model. Values below 10 % are indicative of a good fit (McLaughlin & O'Beirne, 1999).

$$E = \frac{100}{N} \cdot \sum_{1}^{N} \frac{\left|\left(R_{obs} - R_{pre}\right)\right|}{R_{obs}} \qquad \text{Eq. 6-2}$$

6.3. RESULTS AND DISCUSSION

6.3.1 Critical surface tension and surface tension of cheese

The determination of the surface tension and of the critical surface tension of the cheese allows the characterization of the surface of its skin. The surface from the cheese displays values of critical surface and surface tension of 18.33 ± 0.10 mN m^{-1} and 37.79 ± 0.76 mN m^{-1} respectively. The cheese surface is a low-energy surface (<100 mN m^{-1}) and presents a higher dispersive component (29.93 ± 0.41 mN m^{-1}), which shows its ability to participate in non-polar interactions, and a low polar component (7.87 ± 0.37 mN m^{-1}). A surface with these characteristics interacts with liquid primarily by dispersion forces, influencing the effective spreading of the coating on the cheese surface. The compatibility of the polarity (apolar or polar)

of the surface and of the coating may play therefore an important role in the wettability of the surface. The cheese, being very rich in apolar components (e.g. fat) features a significant apolar influence.

6.3.2 Wettability

The wettability of the coating solutions was studied by determining the values of the spreading coefficient (W_s). Wettability is one of the most important properties when evaluating the capacity of a solution to coat a designated surface. In practical terms, the closer the W_s values are to zero, the better a surface will be coated.

Figure 6-1a and 6-1b shows the Pareto charts for chitosan coatings. Chitosan concentration was the parameter which most influenced W_s in both experimental setups (I and II). Also the interaction between chitosan and oil was an influent parameter on the value of W_s, only overtaken in the experimental setup II, by the interaction between chitosan and the mixture glycerol/sorbitol.

In both experimental setups the plasticizer showed approximately the same standardized effect (closer to -5.0), however the replacement of a part of glycerol by the same concentration of sorbitol in experimental setup II changes the standardized effect of some of the factors on W_s. One of the most evident is the presence of the oil, that in experimental setup I show a standardized effect of -10.33, as one of the most influent factors, and in experimental setup II does not have influence in the W_s.

The oil incorporation in the coating formulation with 0.5 % of chitosan increased W_s values (Table 6-2). In fact, the incorporation of oil in the solution of chitosan will form a micellar structure in the presence of Tween 80 as shown in Chapter 5, however in this case, for cheese surface the W_s increase in the presence of oil.

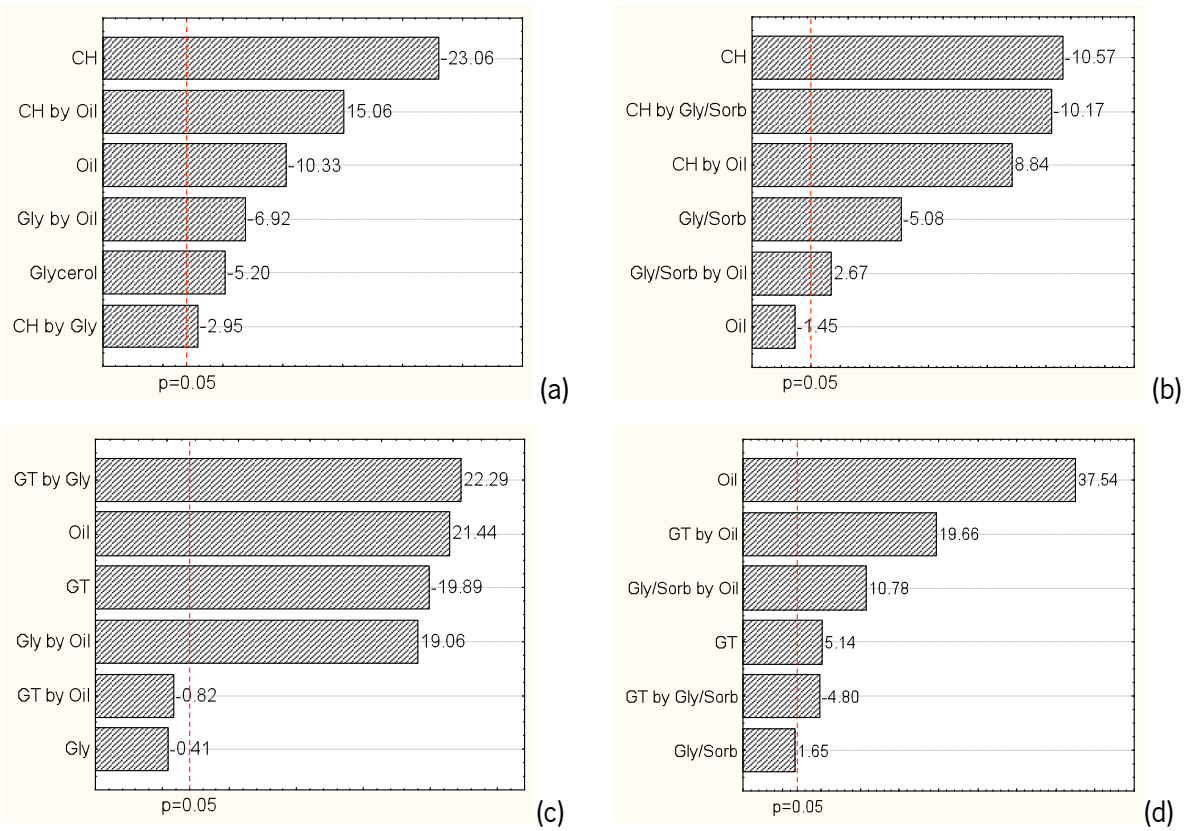

Figure 6-1. Pareto charts of the effects of the experimental setup I (a, c) and II (b, d) of chitosan (a, b) and galactomannan (c, d) coatings/films for spreading coefficient (W_s).

The higher values of W_s of the solutions with lower chitosan concentrations can be explained by the high ratio between the concentration of Tween 80 and the concentration of chitosan. The best W_s values were obtained for the coatings 1, 2 and 6, and without statistically difference between each other (Table 6-2).

For galactomannan coatings the oil presence is the most influent parameter, only overtaken by the interaction between galactomannan and glycerol concentrations (Figure. 6-1c and 6-1d). The presence of oil in galactomannan coatings decreased the values of W_s. The partly hydrophobic surface of the cheese, as explained previously, presents a good adhesion to the coatings containing oil, eventually due to the ability of the solution with oil (more hydrophobic) to interact with the cheese surface (Van Oss, 1995). Moreover, for galactomannan the coatings with higher values of W_s were those containing oil. The coatings formulations with the best W_s values were the coatings 3, 12 and 15 (Table 6-2).

Table 6-2. Formulations used in coatings/films analyses; spreading coefficient (*Ws*) obtained for the tested polysaccharide (Poly) solutions on cheese

Sample	Poly (w/v)	Gly (w/v)	Gly/Sorb (w/v)	Oil (w/v)	Spreading coefficient (W_s)	
					Chitosan*	*G. triacanthos**
1	0.5	0.5		0.0	-28.97±1.62^{a}	-42.94±2.52^{a}
2	0.5	2.0		0.0	-29.81±1.66^{a}	-57.84±4.87^{b}
3	0.5	0.5		0.5	-34.50±1.50^{b}	-37.05±2.59^{c}
4	0.5	2.0		0.5	-35.76±2.99^{bc}	-41.69±2.85$_{ae}$
5	0.5		0.5	0.0	-34.46±2.33^{b}	-49.69±4.03^{d}
6	0.5		2.0	0.0	-29.96±3.10^{a}	-54.79±0.78^{b}
7	0.5		0.5	0.5	-36.62±1.89^{bcd}	-51.01±2.37^{d}
8	0.5		2.0	0.5	-36.49±2.19^{bcd}	-41.93±2.77^{ae}
9	1.5	0.5		0.0	-38.31±2.11^{cde}	-58.97±3.65^{b}
10	1.5	2.0		0.0	-38.95±1.65^{de}	-59.53±3.65^{b}
11	1.5	0.5		0.5	-34.65±2.22^{b}	-59.03±1.86^{b}
12	1.5	2.0		0.5	-40.13±2.84^{e}	-38.76±3.38^{ce}
13	1.5		0.5	0.0	-36.11±1.98^{bc}	-56.12±2.30^{b}
14	1.5		2.0	0.0	-49.56±0.76^{f}	-55.99±1.28^{b}
15	1.5		0.5	0.5	-37.74±2.48^{cde}	-40.16±1.40^{ace}
16	1.5		2.0	0.5	-40.31±2.64^{e}	-41.45±2.59^{ae}

*Values reported are the means ± standard deviations (n=20, 95 % confidence interval, at 21.3 ± 0.5 °C). $^{a-e}$Different superscript letters in the same column indicate a statistically significant difference (Tukey test, p<0.05). Shadowed in grey are the best values for the same group of polysaccharides.

The present results do not meet with the results obtained in Chapter 5 (section 5.3.2.1) where it has been shown that for chitosan film forming solutions the increase of the glycerol concentration have lead to a decrease of the W_s in the studied surfaces (SS316, glass and PMMA), while in this case where cheese surface was used this not happen for chitosan film forming solutions with a chitosan concentration of 1.5 %. Also for galactomannan film forming solutions the increase of oil led to lower W_s values when cheese surface was used, while for the three model surfaces used their increase leads to higher values of W_s. Cheese surface is quite irregular, when compared with the model surfaces used, leading to a great variability and different interactions between the film forming solutions and the cheese surface.

Table 6-3 shows the parameters of the model presented in Equation 6-1 that provided the best fit to the obtained results. In all the cases (experimental setups I and II) the values of R^2 were always higher than 0.80, the values of E were always below 10 % and those of A_f were very close to 1, showing that the model provided a good fit to the data. Casariego et al. (2008) used a similar model to adjust the values of W_s in tomato and carrot also with good results, with values of R^2 higher than 0.85.

Table 6-3. Model equations of spreading coefficient (W_s) and the corresponding quality of the fit evaluation parameters of experimental setup I and II for chitosan and galactomannan coatings

	Model Equations	R^2	E	A_f
Chitosan	W_{sI}=-25.0668–8.9589×CH+1.3695×Gly–15.5969×Oil–1.0602×CH× Gly+10.2515×CH×Oil–4.9808×Gly×Oil	0.93	1.33	1.02
	W_{sII} = -32.6101–27.1886×Oil+4.4548×Gly/Sorb+20.3578×Chi×Oil–7.7341×Chi×Gly/Sorb+ 4.8637×Gly/Sorb×Oil	0.82	1.33	1.02
Galactomannan	W_{sI} = -21.0545-25.7074×GT-19.5232×Gly-7.9445×Oil+13.5889× GT×Gly-1.4960×GT×Oil+23.2367×Gly×Oil	0.95	2.12	1.00
	W_{sII} = -50.1644-1.7511×GT+0.0834×Gly-10.6013×Oil-1.7183×GT× Gly+21.1178×GT×Oil+7.7192×Gly×Oil	0.87	4.10	1.04

*6.3.3 Water vapour permeability (*WVP*)*

Figure 6.2 shows that oil is one of the most important factors influencing the value of *WVP*. Also plasticizer concentration is one of the factors with more significance. However, also chitosan concentration presents a great influence for films with sorbitol (Figure 6.2b).

Table 6-4 shows that *WVP* increases with the increase of plasticizer concentration; this observation is common to both experimental setups. Higher concentrations of plasticizer favour the adsorption of water molecules, which is mainly attributed to the predisposition of plasticizers to form hydrogen bonds. Plasticizers and its plasticizing action change the polymer network creating mobile regions with greater interchain distances, promoting water clustering by competing with water at active sites of the polymer matrix and reducing intermolecular hydrogen bonding between polysaccharide molecules (Olivas & Barbosa-Cánovas, 2008). The results are in agreement with the obtained in Chapter 5.

Experimental setup II (Table 6-4) shows that the incorporation of sorbitol in detriment of part of the glycerol is an alternative to reduce the effect of glycerol in the increase of *WVP* for chitosan and galactomannan films. Results may be explained by the free volume theory: plasticizers increase the free volume of polymer structures or the molecular mobility of polymer molecules. In this case the decrease of *WVP* may be attributed to the larger size and relatively lower hygroscopicity of sorbitol compared to glycerol that might reduce the amount of water entrapped in the film matrix; such film would therefore have a higher effective concentration of polysaccharide, thus reducing water mobility and, consequently, reducing *WVP.* Previous works has shown that edible films containing sorbitol as plasticizer have lower *WVP* values than those containing glycerol (Fairley et al., 1996; Chick & Ustunol, 1998; Olivas & Barbosa-Cánovas, 2008).

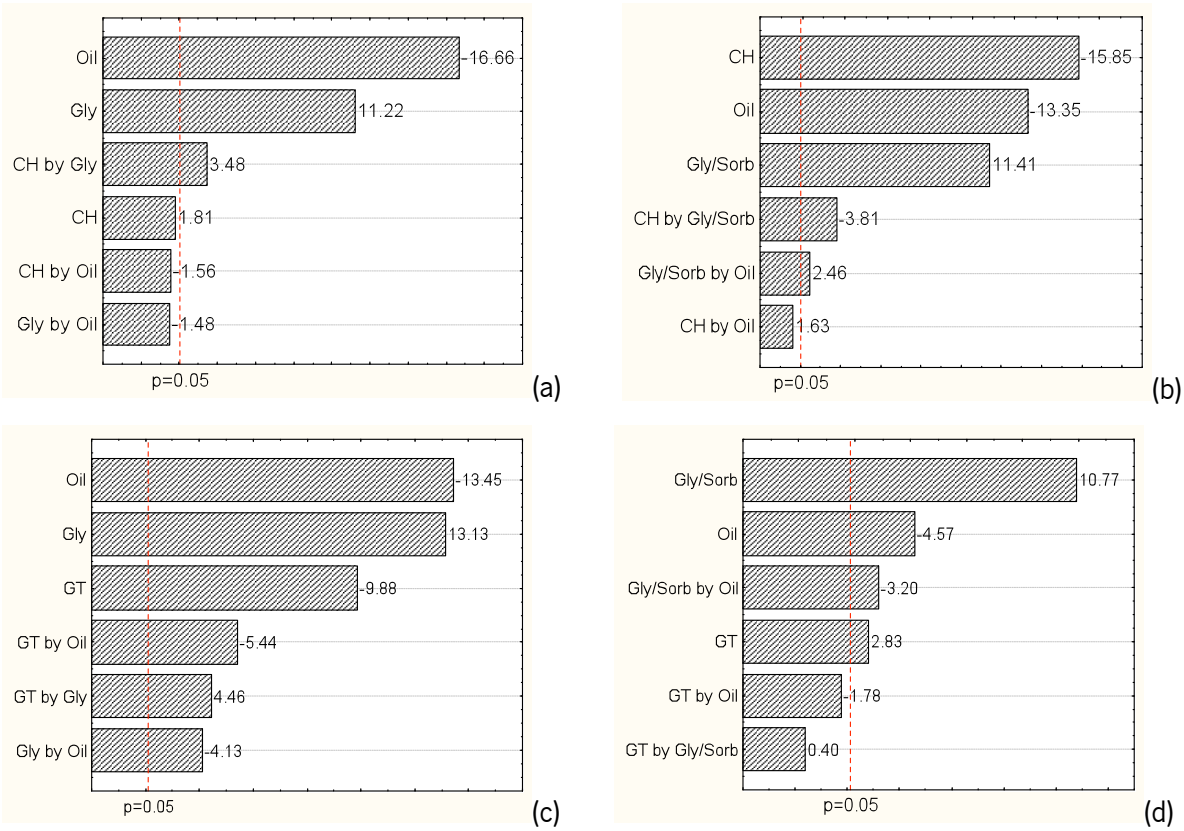

Figure 6-2. Pareto charts of the effects of the experimental setup I (a, c) and II (b, d) of chitosan (a, b) and galactomannan (c, d) films for water vapour permeability (*WVP*).

The incorporation of oil decreased the *WVP* for both polysaccharides. The presence of the oil blended with polysaccharide changes the film properties decreasing the water affinity (Wong et al., 1992; Bozdemir et al., 2003; Vargas et al., 2009). The influence of glycerol and oil are in agreement with the observed in section 5.3.1.5 and 5.3.2.5 (Chapter 5), for chitosan and galactomannan films, respectively.

When the model equation (Eq. 6-1) was fitted to the *WVP* experimental data a good fit, with R^2 above 0.88, E below 10 % and A_r very close to 1, was obtained in all cases (Table 6-5).

Table 6-4. Values of thickness and water vapour permeability (*WVP*) for chitosan and galactomannan films

Sample	Chitosan		Galactomannan	
	Thickness (mm)	$WVP \times 10^{11}$ (g m^{-1} s^{-1} Pa^{-1})	Thickness (mm)	$WVP \times 10^{11}$ (g m^{-1} s^{-1} Pa^{-1})
1	0.060 ± 0.004^{ac}	8.19 ± 0.55^{a}	0.058 ± 0.004^{a}	8.30 ± 0.33^{a}
2	0.062 ± 0.003^{ab}	10.30 ± 0.79^{b}	0.060 ± 0.005^{a}	9.24 ± 0.18^{b}
3	0.059 ± 0.002^{a}	6.87 ± 0.29^{c}	0.059 ± 0.005^{a}	7.70 ± 0.20^{c}
4	0.067 ± 0.002^{b}	7.08 ± 0.22^{cd}	0.060 ± 0.007^{ab}	8.36 ± 0.21^{a}
5	0.060 ± 0.004^{ac}	7.65 ± 0.48^{ad}	0.056 ± 0.003^{a}	6.86 ± 0.51^{de}
6	0.056 ± 0.001^{cd}	8.36 ± 0.33^{ae}	0.064 ± 0.003^{b}	8.50 ± 0.33^{a}
7	0.052 ± 0.003^{d}	6.26 ± 0.35^{c}	0.058 ± 0.004^{a}	6.84 ± 0.31^{de}
8	0.053 ± 0.002^{d}	7.68 ± 0.28^{a}	0.064 ± 0.002^{a}	7.78 ± 0.18^{c}
9	0.067 ± 0.001^{b}	8.90 ± 0.47^{e}	0.065 ± 0.005^{a}	7.23 ± 0.14^{d}
10	0.066 ± 0.001^{b}	10.60 ± 0.09^{b}	0.074 ± 0.005^{b}	9.30 ± 0.34^{b}
11	0.063 ± 0.001^{a}	5.65 ± 0.62^{f}	0.071 ± 0.003^{b}	6.11 ± 0.53^{e}
12	0.060 ± 0.002^{a}	8.37 ± 0.39^{a}	0.074 ± 0.007^{b}	7.11 ± 0.22^{c}
13	0.071 ± 0.005^{b}	6.08 ± 0.36^{f}	0.071 ± 0.003^{b}	7.30 ± 0.26^{c}
14	0.067 ± 0.004^{ab}	7.32 ± 0.48^{a}	0.071 ± 0.002^{b}	9.11 ± 0.08^{b}
15	0.053 ± 0.001^{d}	4.04 ± 0.10^{g}	0.063 ± 0.009^{b}	6.99 ± 0.31^{de}
16	0.055 ± 0.001^{cd}	5.63 ± 0.21^{f}	0.071 ± 0.002^{b}	7.98 ± 0.21^{c}

$^{a-e}$ Means in the same column with different superscripts are significantly different ($p<0.05$).

Table 6-5. Model equations of water vapour permeability (*WVP*) and the corresponding quality of the fit evaluation parameters of experimental setup I and II for chitosan and galactomannan films

	Model Equations	R^2	E	A_f
Chitosan	WVP_I= 8.2713-0.3671×CH+0.5764×Gly–3.3336×Oil+ 0.6995×CH×Gly –0.9412×CH×Oil-0.5957×Gly×Oil	0.88	5.20	1.05
	WVP_{II} = 8.1478–1.6040×CH+0.4111×Gly/Sorb-2.1366×Oil+ 0.2368×CH×Gly/Sorb – 1.6551×CH×Oil+0.7134×Gly/Sorb×Oil	0.95	1.30	1.01
Galactomannan	WVP_I =8.3351-1.1004×GT+0.5152×Gly+0.7986×Oil+ 0.5510×GT×Gly-2.0158×GT×Oil-1.0211×Gly×Oil	0.96	1.02	1.50
	WVP_{II}=6.0408+0.4762×GT+1.0615×Gly+1.0448×Oil+ 0.0647×GT×Gly-0.85952×GT×Oil-1.03191×Gly×Oil	0.91	0.55	1.01

6.3.4 Oxygen Permeability (O_2P*)*

Oxygen is the key factor that might cause oxidation and which initiates several unwanted changes in foods such as odour, colour, flavour and nutrients deterioration. Films providing a proper barrier to oxygen can help improving food quality and extending food shelf-life.

Figure 6-3a and 6-3b shows that for chitosan films the polysaccharide and plasticizer concentrations are the most significant factors in O_2P, as shown by Pareto charts. The interaction of oil with chitosan is also a significant factor in both cases. Table 6.6 shows that the increase of plasticizer concentration provoked a significant decrease in the O_2P values. In this case the increase of the number of hydroxyl groups due to a higher plasticizer concentration enhanced the effect of hydrogen bounding, and increased the cohesive energy density. The cohesive energy density is a measure of the polarity of a polymer and of the energy binding the polymer chains together. In general, the higher the polymer cohesive energy density, the more difficult it is for the polymer chains to open and allow a permeant to pass (polar permeants such as water being an exception to this rule) (Miller & Krochta, 1997).

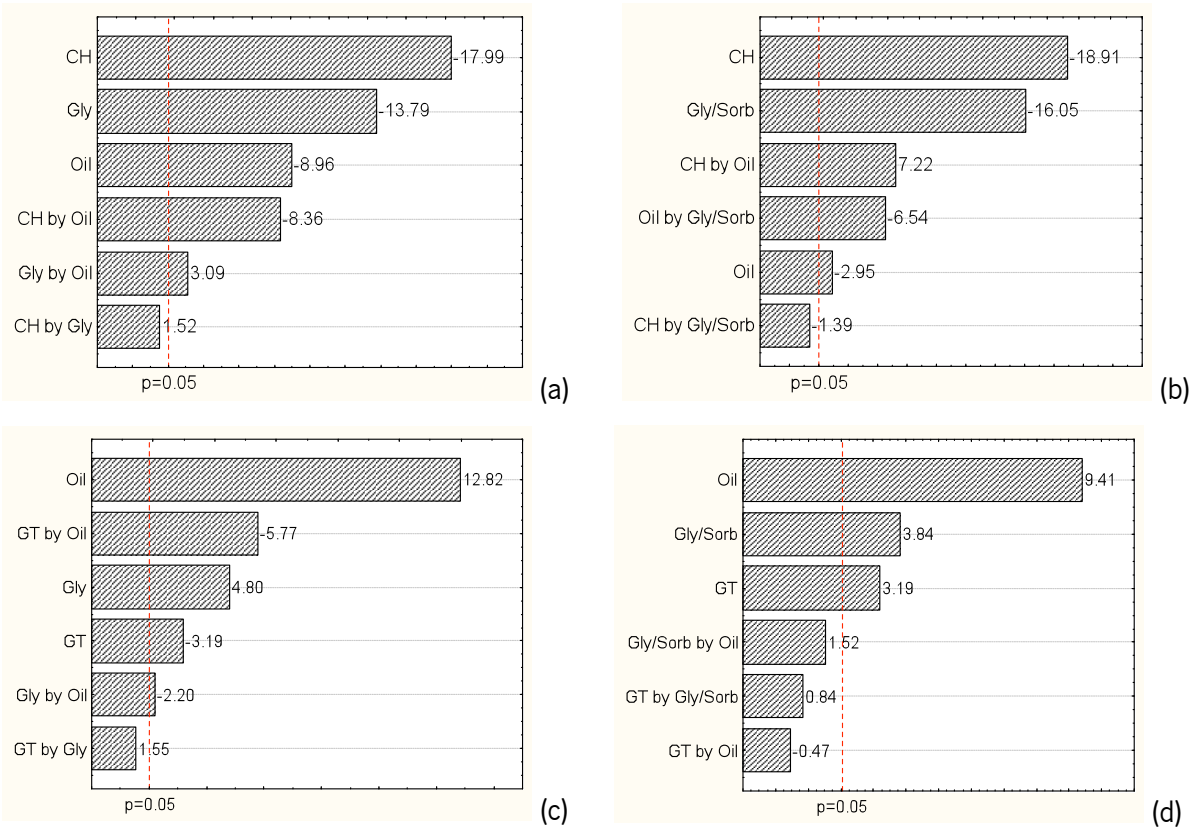

Figure 6-3. Pareto charts of the effects of the experimental setup I (a, c) and II (b, d) of chitosan (a, b) and galactomannan (c, d) films for oxygen permeability (O_2P).

For galactomannan films the oil presence shows to be the most important factor (Figure 6-3c and 6-3d). The higher values of O_2P obtained for films containing oil when compared to similar concentrations of galactomannan and glycerol (Table 6-6) can be explained by the presence of oil droplets inserted between galactomannan chains, which interrupt the film matrix and possibly contribute to develop a more open structure. Galactomannan and plasticizer concentrations are also influent factors on O_2 permeability. In this case the addition of plasticizer increases the free volume of the film leading to an increase of permeability values. Moreover, the hydrophilicity of the glycerol molecule, which favours the adsorption of water molecules, might enhance the solubility of the gases in the films, increasing their permeability (Miller & Krochta, 1997).

Table 6-6. Values of oxygen permeability (O_2P) and carbon dioxide permeability (CO_2P) for chitosan and galactomannan films

Sample	Chitosan		Galactomannan	
	$O_2P \times 10^{-15}$ (g $Pa^{-1}s^{-1}m^{-1}$)	$CO_2P \times 10^{-15}$ (g $Pa^{-1}s^{-1}m^{-1}$)	$O_2P \times 10^{-15}$ (g $Pa^{-1}s^{-1}m^{-1}$)	$CO_2P \times 10^{-15}$ (g $Pa^{-1}s^{-1}m^{-1}$)
1	7.61±0.38^{a}	35.65±1.99^{ac}	2.04±0.81^{a}	23.37±4.62^{af}
2	5.15±0.55^{b}	23.42±2.92^{b}	3.02±0.59^{ac}	33.15±2.23^{b}
3	7.46±0.49^{a}	36.41±3.52^{ac}	5.87±0.57^{b}	32.48±2.25^{b}
4	5.11±0.09^{b}	27.53±3.39^{b}	6.10±0.77^{b}	38.76±1.65^{c}
5	8.96±0.51^{c}	31.32±3.06^{a}	2.52±0.61^{a}	19.83±4.29^{a}
6	7.45±0.86^{ae}	30.21±3.82^{ab}	2.72±0.83^{a}	30.00±1.92^{b}
7	9.19±0.54^{c}	36.15±2.72^{c}	5.69±0.63^{be}	24.47±1.05^{a}
8	4.73±0.58^{d}	18.78±2.41^{d}	8.07±1.40^{d}	34.28±1.49^{b}
9	6.29±0.52^{e}	26.68±1.75^{b}	2.22±0.72^{a}	11.41±1.12^{d}
10	3.46±0.62^{f}	39.84±1.71^{e}	3.82±0.20^{c}	22.78±1.44^{af}
11	2.51±0.51^{f}	34.63±3.61^{ac}	3.97±0.24^{c}	15.16±2.54^{e}
12	1.65±0.53^{g}	59.80±3.42^{f}	4.69±0.28^{e}	17.12±3.96^{feg}
13	5.59±0.41^{b}	16.00±0.55^{d}	3.30±0.30^{c}	11.44±1.75^{g}
14	2.88±0.64^{f}	34.36±5.95^{ac}	5.10±0.79^{b}	25.46±2.78^{a}
15	6.58±0.23^{e}	15.66±1.24^{dg}	6.93±1.79^{b}	14.26±3.70^{g}
16	3.35±0.68^{f}	21.24±3.55^{g}	9.17±2.58^{d}	20.54±4.75^{a}

$^{a-g}$ Means in the same column with different superscripts are significantly different (p<0.05).

The lowest value of O_2P was obtained with the film made with 1.5 % of chitosan, 2.0 % of glycerol and 0.5 % of oil (sample 12). The obtained values are higher than those reported by Caner et al. (1998) were a lower amount of plasticizer was added, who reported values ranging between 0.26 × 10^{15} g m $Pa^{-1}s^{-1}m^{-2}$ and 0.05 × 10^{15} g m $Pa^{-1}s^{-1}m^{-2}$ for films containing 0.5 % and 0.25 % of polyethylene glycol 400 per gram of chitosan.

The model fitted to O_2P data (Eq. 6-1) presents an acceptable value for R^2 (above 0.78) and a value of A_f close to 1; also the value of E range between is lower than 10-20 % indicating a good the fit between the observed and predicted values (Table 6-7).

Table 6-7. Model equations for oxygen permeability (O_2P) and the corresponding quality of the fit evaluation parameters of experimental setup I and II for chitosan and galactomannan films

	Model Equations	R^2	E	A_f
Chitosan	O_2P_I = 9.7308–1.9027×CH–2.0779×Gly+0.8177×Oil+ 0.3164×CH×Gly-5.2328×CH×Oil+1.2885×Gly×Oil	0.95	6.37	1.07
	O_2P_{II} = 11.8731–4.0574×CH-0.7635×Gly/Sorb–2.2079×Oil- 0.3168×CH×Gly/Sorb+4.9187×CH×Oil-2.9722×Gly/Sorb×Oil	0.93	8.40	1.09
Galactomannan	O_2P_I=1.6885+0.0069×GT+0.4858×Gly+10.4057×Oil+ 0.3788×GT×Gly-4.2783×GT×Oil-8.161×Gly×Oil	0.88	1.00	0.47
	O_2P_{II}=1.6007+0.9764×GT+0.1840×Gly+6.7474×Oil+ 0.4835×GT×Gly-0.8145×GT×Oil+1.7427×Gly×Oil	0.78	4.91	1.05

6.3.5 Carbon dioxide permeability (CO_2P*)*

Figure 6-4 shows the Pareto charts of the effects for chitosan and galactomannan films for the experimental setups I and II. In the case of chitosan films, the interaction between chitosan and glycerol, and chitosan alone in experimental setup I, and the interaction of chitosan and glycerol/sorbitol, as weel as the interaction of chitosan and oil in the experimental setup II are the most significant factors affecting the value of CO_2P (Figure 6-4a and 6-4b). These results show that plasticizers have different influences on the values of O_2P and CO_2P. The plasticizers increase the cohesive energy density and the free volume of polymer structures. The influence of the cohesive energy density in the values of O_2P has been previously discussed, and it can also explain the decrease of the CO_2P values for concentrations of 0.5 % of chitosan. For higher concentrations of chitosan (1.5 %) the free volume of polymer seems to be the most important

factor, leading to an increase of CO_2P with the increase of the plasticizer concentration. These observations are in line with those of Miller and Krochta (1997), who reported that the diffusion coefficient and the permeability coefficient both increase with the increase in free volume for carbon dioxide in various polymers.

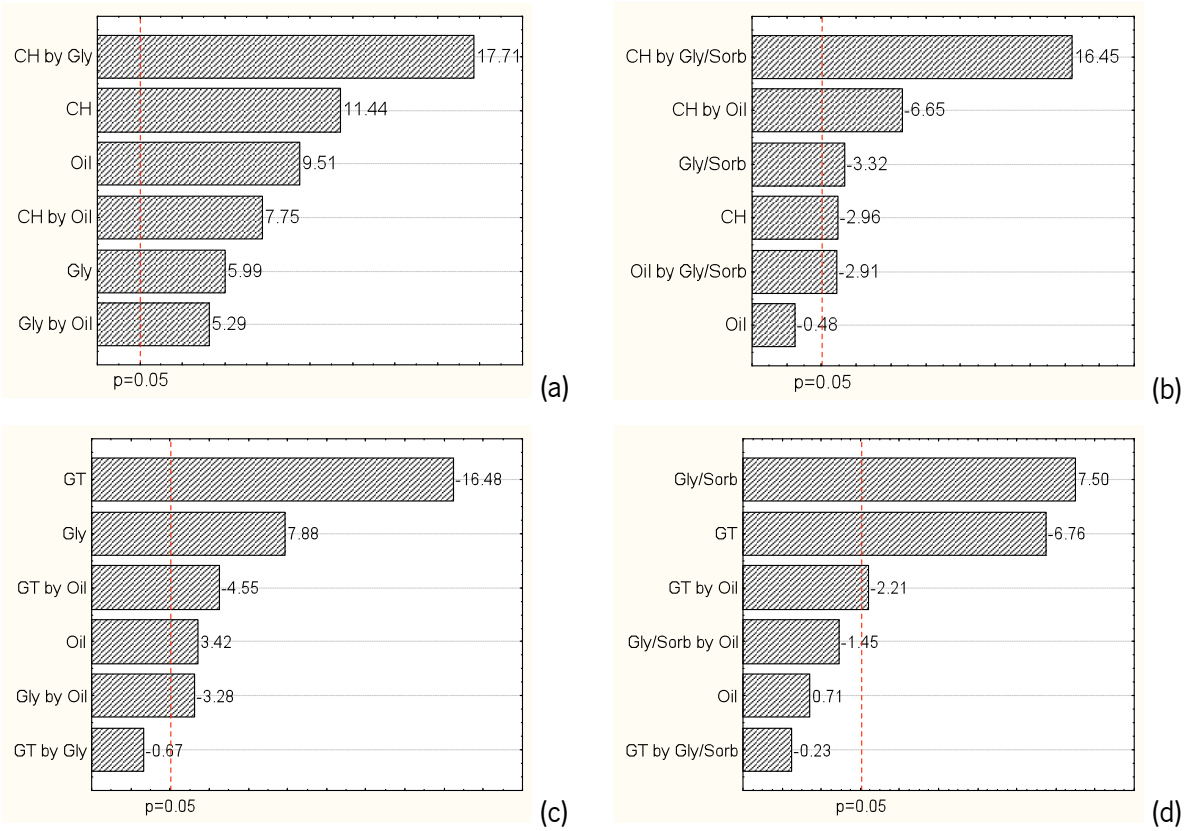

Figure 6-4. Pareto charts of the effects of the experimental setup I (a, c) and II (b, d) of chitosan (a, b) and galactomannan (c, d) films for carbon dioxide permeability (CO_2P).

For galactomannan films the polysaccharide and plasticizers concentrations present to be the most influent factors affecting the CO_2P with (Figure 6-4c and 6-4d). The increase of polysaccharide concentration lead to a decrease of the CO_2P while for the increase of plasticizer concentration is observed an increase of the values (Table 6-6).

The higher values obtained for CO_2P when compared with the oxygen permeability can be explained by the solubility of these gases in water. The CO_2 is approximately 35 times more

soluble than O_2 in water, and this is the reason why this gas diffuses much faster, therefore increasing its permeability as observed in this study, at 50 % RH (Mujica-Paz & Gontard, 1997).

The results are in agreement with the values obtained for chitosan films with fatty acids, with values ranging between 4.09 and 22.69 × 10^{15} g m Pa^{-1} s^{-1} m^{-2}, depending on the fatty acid used (Wong et al., 1992).

The fitting of the model equation (Eq. 6-1) to the experimental values of CO_2P shows quite good results, with values of R^2 above 0.74, E below 10 % and A_f very close to 1 (Table 6-8).

Table 6-8. Model equations for carbon dioxide permeability (CO_2P) and the corresponding quality of the fit evaluation parameters of experimental setup I and II for chitosan and galactomannan films

	Model Equations	R^2	E	A_f
Chitosan	CO_2P_I= 52.1856–20.8821×CH–18.7193×Gly–23.9486×Oil+ 19.0938×CH×Gly+25.0603×CH×Oil+11.4097×Gly×Oil	0.95	3.14	1.03
	CO_2P_{II} = 45.6805–21.8885×CH-20.4924×Gly/Sorb+32.6308×Oil +20.2395×CH×Gly/Sorb-24.5676×CH×Oil –7.1642×Gly/Sorb×Oil	0.88	8.99	1.09
Galactomannan	CO_2P_I=24.0909-10.1212×GT+7.8020×Gly+33.6568×Oil- 0.8353×GT×Gly-17.0416×GT×Oil-8.1609×Gly×Oil	0.91	1.04	3.70
	CO_2P_{II}=17.1420-5.3104×GT+8.0046×Gly+19.2769×Oil- 0.3898×GT×Gly-11.2969×GT×Oil-4.9389×Gly×Oil	0.74	5.78	1.06

6.3.6 Opacity

Opacity means a smaller transparency, important to control the incidence of light on the cheese, being a relevant property since it has a direct impact on the appearance of the coated product. For both chitosan and galactomannan films the polysaccharide concentration is the most important factor affecting their opacity, followed by plasticizer concentration (Figure 6-5). The increase of polysaccharide concentration originates a film matrix with a stronger polymer network resulting on higher values of opacity; on the other hand, the increase of glycerol concentration leads to an increase of the free volume of the polymer network, as explained before (Chapter 5), thus increasing the mobility of the polymer chains and decreasing the opacity by permitting a better penetration of the light. Table 6-9 shows that opacity values are higher in films containing oil. The increase of opacity registered when oil was added was a result from the presence of lipid droplets formed during the coating formulation that are dispersed in the emulsion and distributed in the polymer matrix after the film is formed. The fitting of the model equation (Eq. 6-1) to the experimental values of opacity shows very good results, with values of R^2 above 0.95, E below 10 % and an A_f value of 0.98 (Table 6-10).

*6.3.7 Tensile strength (*TS*) and elongation-at-break (*EB*)*

Figure 6-6 shows Pareto charts of effects of the factors affecting *TS*. For the two kinds of films (chitosan and galactomannan) polysaccharide concentration, plasticizer concentration and the interaction between both are the most significant factors. The increase of polysaccharide concentration increased the *TS* values; this effect may be explained by the formation of a stronger gel network, where the polysaccharide molecules are closer, forming a more coherent film structure, and reducing the absorption of water molecules. The increase of plasticizer concentration has great influence on the values of *TS* for films explained by the plasticizing effect of glycerol and sorbitol that changes the polymer network and creates more mobile regions with larger interchain distances, thus decreasing *TS* (Caner et al., 1998; Ziani et al., 2008).

Table 6-9. Values of opacity, tensile strength (*TS*) and elongation-at-break (*EB*) for chitosan and galactomannan films

Sample	Chitosan			Galactomannan		
	Opacity	*TS* (MPa)	*EB* (%)	Opacity	*TS* (MPa)	*EB* (%)
1	2.74±0.20^{a}	0.63±0.01^{a}	58.62±7.83^{a}	5.23±0.20^{a}	0.63±0.01^{a}	58.62±7.83^{a}
2	2.44±0.13^{b}	0.15±0.01^{b}	81.24±7.53^{b}	3.56±0.21^{b}	0.15±0.01^{b}	81.24±7.53^{b}
3	3.22±0.33^{c}	0.94±0.17^{b}	68.55±6.53^{a}	7.72±0.11^{c}	0.94±0.17^{c}	68.55±6.53^{a}
4	2.78±0.32^{b}	0.39±0.04^{b}	119.52±4.45^{b}	3.83±0.21^{b}	0.39±0.04^{d}	119.52±4.45^{c}
5	3.03±0.21^{b}	0.70±0.11^{b}	78.92±9.75^{b}	5.11±0.50	0.70±0.11^{c}	78.92±9.75^{b}
6	3.85±0.38^{b}	0.21±0.05^{b}	166.70±7.36^{b}	3.54±0.26^{b}	0.21±0.05^{b}	166.70±7.36^{d}
7	2.28±0.26^{b}	1.58±0.14^{b}	102.03±4.63^{b}	7.34±0.76^{b}	1.58±0.14^{e}	102.03±4.63^{e}
8	2.85±0.22^{b}	0.43±0.06^{b}	181.02±5.21^{b}	4.14±0.53^{b}	0.43±0.06^{d}	181.02±5.21^{d}
9	3.94±0.44^{d}	13.72±0.43^{c}	89.68±4.99^{c}	11.30±1.33^{d}	13.72±0.43^{f}	89.68±4.99^{b}
10	2.99±0.12^{e}	1.22±0.20^{a}	97.45±3.77^{a}	6.10±0.09^{e}	1.22±0.20^{e}	97.45±3.77^{e}
11	4.35±0.48^{d}	12.71±1.22^{c}	81.00±4.99^{c}	12.77±2.62^{d}	12.71±1.22^{f}	81.00±4.99^{b}
12	3.60±0.20^{c}	0.99±0.10^{d}	89.60±2.05^{d}	7.33±0.64^{c}	0.99±0.10^{c}	89.60±2.05^{b}
13	4.08±0.17^{b}	12.08±0.58^{c}	81.97±8.77	11.20±2.09^{b}	12.08±0.58^{f}	81.97±8.77^{b}
14	3.75±0.18^{b}	8.47±0.61^{a}	109.97±9.83	6.38±1.26^{b}	8.47±0.61^{g}	109.97±9.83^{e}
15	4.58±0.49^{b}	10.46±1.92^{c}	60.58±6.74	11.87±2.1^{b}	10.46±1.92^{g}	60.58±6.74^{a}
16	3.55±0.15^{b}	3.18±0.43^{d}	124.20±8.79	7.58±1.54^{b}	3.18±0.43^{h}	124.20±8.79^{c}

$^{a–e}$ Means in the same column with different superscripts are significantly different (p<0.05).

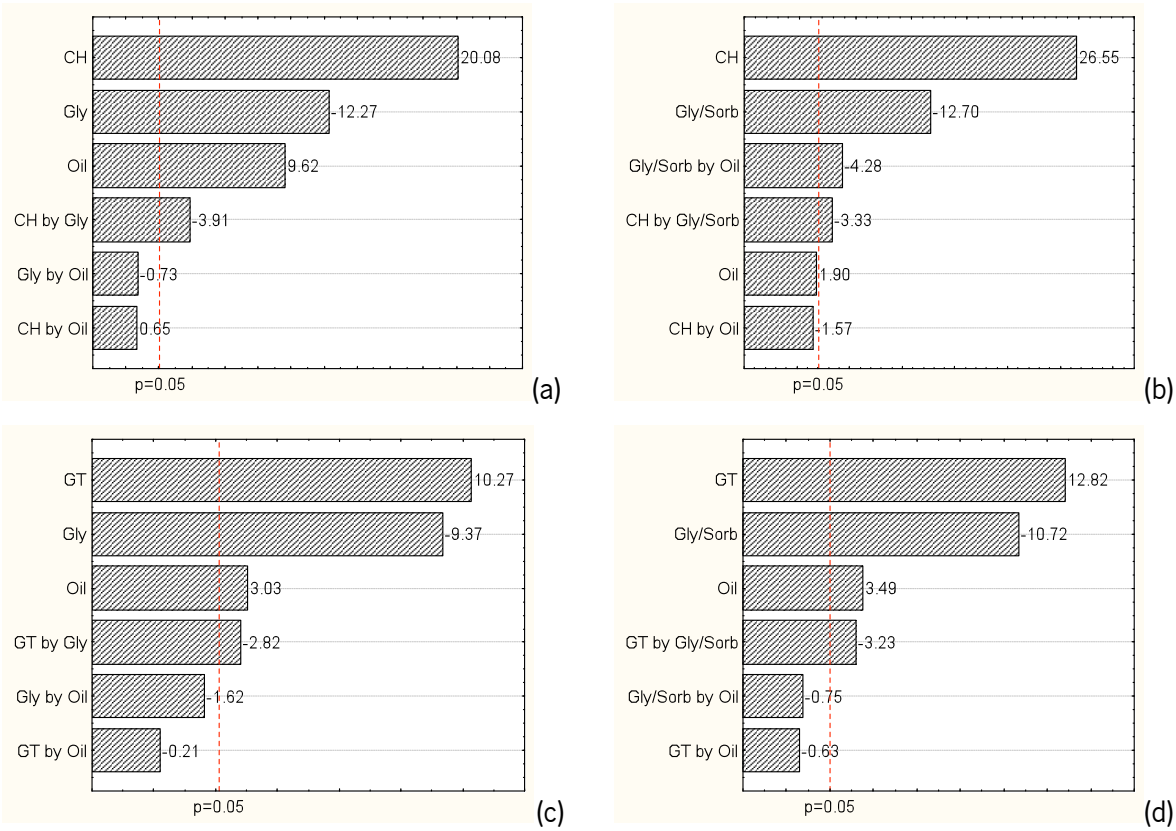

Figure 6-5. Pareto charts of the effects of the experimental setup I (a, c) and II (b, d) of chitosan (a, b) and galactomannan (c, d) coatings/films for opacity.

Table 6-10. Model equations for opacity and the corresponding quality of the fit evaluation parameters of experimental setup I and II for chitosan and galactomannan films

	Model Equations	R^2	E	A_f
Chitosan	$Opacity_I$ = 2.2498+1.1545×CH-0.1100×Gly+0.8488×Oil–0.2321×CH× Gly+0.1151×CH×Oil-0.0868×Gly×Oil	0.95	2.61	0.98
	$Opacity_{II}$= 2.5467+1.1261×CH-0.0394×Gly/Sorb+0.8146×Oil–0.1484×CH×Gly/Sorb–0.2100×CH×Oil-0.3819×Gly/Sorb×Oil	0.96	2.56	0.98
Galactomannan	$Opacity_I$=2.9111+6.4675×GT-0.5958×Gly+5.22271×Oil-1.5973×GT× Gly-0.3572×GT×Oil-1.8354×Gly×Oil	0.92	1.04	3.46
	$Opacity_{II}$=3.1554+6.1509×GT-0.7661×Gly+3.9149×Oil-1.4055×GT× Gly-0.8195×GT×Oil-0.6521×Gly×Oil	0.83	1.05	4.51

For elongation-at-break (*EB*) the most significant is the plasticizer concentration (Figure 6-7). The increase of the plasticizer concentration leads to an increase of *EB*. Plasticizers interfere with polysaccharide chains and by decreasing intermolecular forces, they reduce the rigidity of the film structure and increase the polymer mobility, thus facilitating film elongation (Srinivasa et al., 2007). The results are in agreement with results obtained in Chapter 5 for chitosan and galactomannan films (section 5.3.2.6 and 5.3.3.6, respectively).

The fitting of the model equation (Eq. 6-1) to the experimental values of *TS* and *EB* shows a good value of R^2 above 0.98; however the values of A_f and *E* present values distant from the optimum (Table 6-11 and Table 6-12) indicating that the fit between the observed and predicted values is not as close as for the other properties tested in the present work.

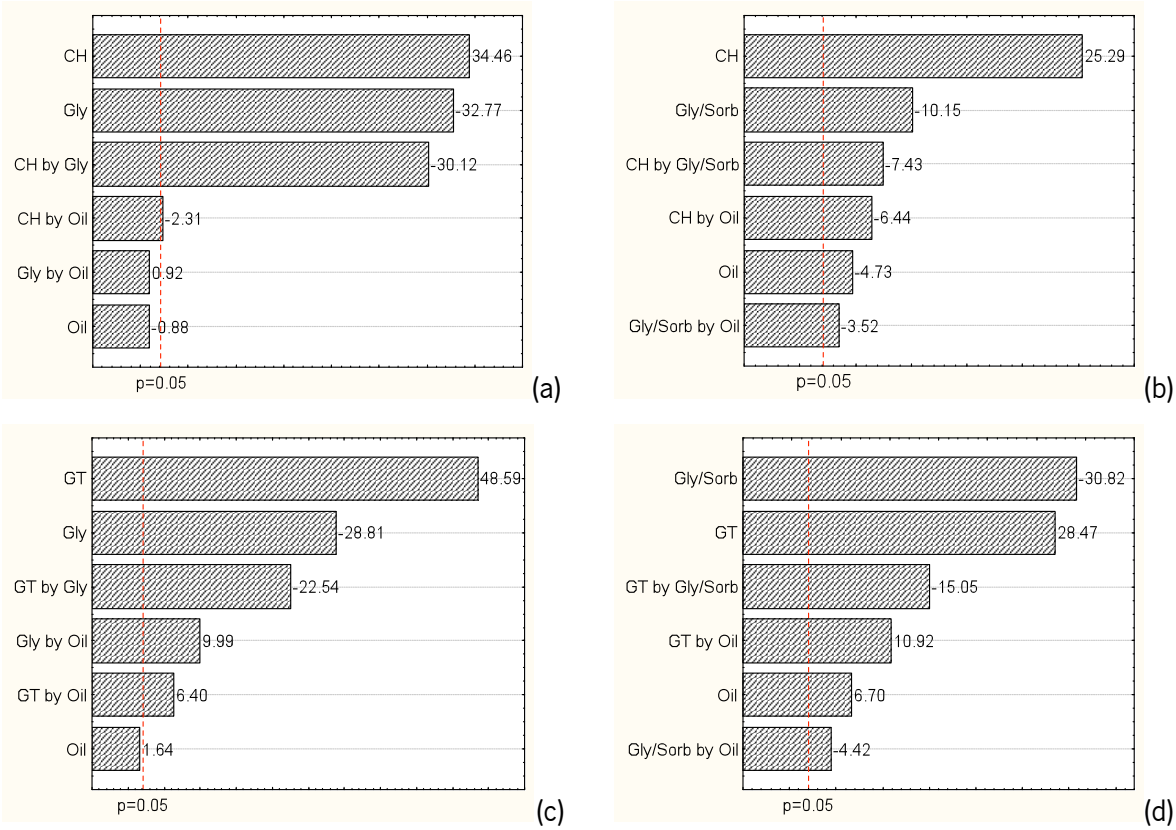

Figure 6-6. Pareto charts of the effects of the experimental setup I (a, c) and II (b, d) of chitosan (a, b) and galactomannan (c, d) films for tensile strength.

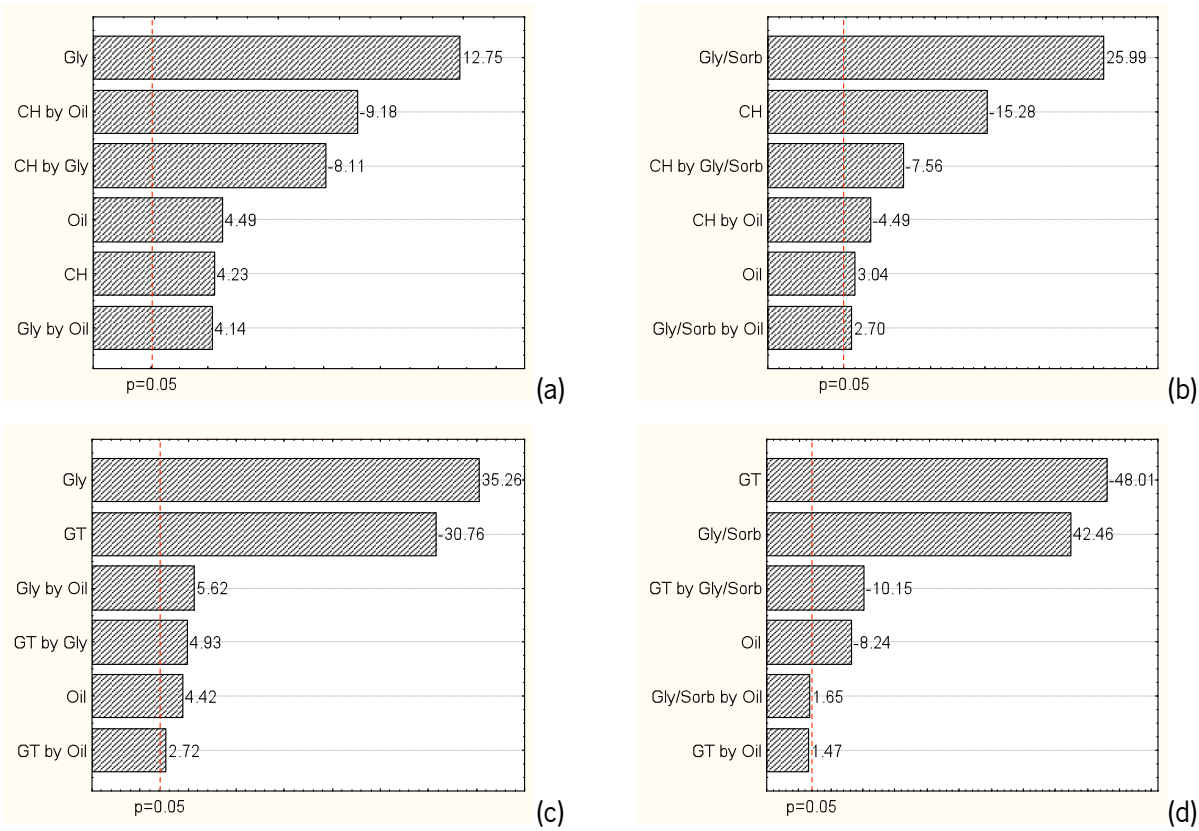

Figure 6-7. Pareto charts of the effects of the experimental setup I (a, c) and II (b, d) of chitosan (a, b) and galactomannan (c, d) films for elongation-at-break.

Table 6-11. Model equations for tensile strength (*TS*) and the corresponding quality of the fit evaluation parameters of experimental setup I and II for chitosan and galactomannan films

	Model Equations	R^2	*E*	A_f
Chitosan	TS_I = -7.4056+16.7415×CH+3.4071×Gly+0.8487×Oil–7.7304×CH× Gly-1.7801×CH×Oil+0.4729×Gly×Oil	0.99	17.97	1.26
	TS_{II} = -6.5835+ 13.6604×CH+1.7006×Gly/Sorb+8.6811×Oil-3.0689×CH×Gly/Sorb-7.9805×CH×Oil-2.9042×Gly/Sorb×Oil	0.98	>20	1.91
Galactomannan	TS_I= -1.6502+1.10161×GT+0.57792×Gly-7.6825×Oil-4.1507×GT×Gly+3.5357×GT×Oil+3.6797×Gly×Oil	0.98	15.6	1.17
	TS_{II}= 1.9171+6.06256×GT+0.3514×Gly-2.2129×Oil-2.8538×GT×Gly+6.2101×GT×Oil-1.6745×Gly×Oil	0.97	10.2	1.11

Table 6-12. Model equations for elongation-at-break (*EB*) and the corresponding quality of the fit evaluation parameters of experimental setup I and II for chitosan and galactomannan films

	Model Equations	R^2	E	A_r
Chitosan	$EB_{(I)}$=21.6055+47.4768×CH+29.2000×Gly+56.2840×Oil–19.0687×CH×Gly– 64.7540×CH×Oil+19.4520×Gly×Oil	0.89	5.72	1.06
	$EB_{(II)}$= 56.6677+4.4807×CH+63.6467×Gly/Sorb+ 37.3700×Oil–25.0533×CH×Gly/Sorb–44.5960×CH×Oil+17.8880×Gly/Sorb×Oil	0.95	5.54	1.06
Galactomannan	$EB_{(I)}$=55.8686-39.4752×GT+12.4769×Gly-19.6774×Oil+6.2254×GT× Gly+10.3098×GT×Oil+14.1828×Gly×Oil	0.97	13.0	1.13
	$EB_{(II)}$= 34.1429-22.7700×GT+28.4836×Gly/Sorb-19.4805×Oil–9.4631×GT×Gly/Sorb+4.1164×GT×Oil+3.0771×Gly/Sorb×Oil	0.99	4.27	1.05

6.3.7 Criteria for choosing a coating

When choosing an adequate coating composition for a food product, exist a number of criteria that should be met. The coating adhesion and durability are important factors to coating effectiveness. The coating must adhere to the food surface during the application and should be able to maintenance during the storage and transportation. The wettability is a parameter that defines the ability of a coating to be uniformly distributed on the food surface (Lin & Zhao, 2007).

Other factors, can also affect the efficiency of the coating, such as transport properties (permeabilities), opacity and mechanical properties; these must also be considered in order to increase the coating performance. The application of the coatings on cheese, not guarantee that coatings will have the same properties of the films cast in plates, however they can be representative of the films properties behaviour. They must be tailored to ensure the appropriate affinity between the coating and the food to be coated.

Being so, the effectiveness of edible coatings for cheese preservation depends, in a first stage, on the control of the wettability of the coating in order to ensure a uniformly coated surface, and in a

second stage in other factors that can also affect the effectiveness of the coating. These should be considered in order to:

1. Decrease the water loss of the cheese (i.e, lower *WVP* values);

2. Decrease O_2 permeability (i.e. lower O_2P values), once oxygen in contact with the cheese contributes to the oxidation of fats and to the growth of undesirable microorganisms (Robertson, 2006);

3. Increase the shelf-life of cheese, by increasing the lag-phase for the growth of coliforms (and other Gram-negative spoilage bacteria), yeasts and moulds (Robertson, 2006), i.e. high CO_2P values. However, some works have shown that there are advantages and disadvantages both for low and high CO_2P values (Papaioannou et al., 2007), thus justify the choice for an intermediate one;

4. Decrease the light incidence in the cheese (light promotes fat oxidation) (Robertson, 2006), i.e. high values of opacity;

5. Increase the resistance of the coating, appropriate mechanical properties can also be useful for cheese protection, reducing the bruising and breakage and thus improving food integrity.

Having in mind the criteria presented above, it is possible to select the best values of wettability, and then the properties that allow achieving the ideal performance described: low *WVP* and O_2P and higher CO_2P, opacity, *TS* and *EB*.

For chitosan coatings/films, the lower W_s values were obtained for coatings 1, 2 and 6 (Table 6-2). For the films corresponding to these coating formulations, the lower *WVP* values were obtained for coating 1 and 6 (Table 6-4) and for these O_2P and CO_2P do not present significant statistically differences, however the film 6 present at the same time the higher opacity and elongation-at-break values. Being so, the coating/film with the formulation of 0.5 % of chitosan and 2.0 % of glycerol/sorbitol was been chosen as the best chitosan coating.

Formulations 3, 12 and 15 present the lower W_s values (Table 6-2) for galactomannans coatings, being the films obtained from formulations 12 and 15 those that present the lower *WVP* values (Table 6-4). Formulation 12 was finally chosen based in its lower O_2P values. In addition the formulation 12 presented higher CO_2P and *EB* values than formulation 15. In this way the coating/film with a formulation of 1.5 % of galactomannan, 2.0 % of glycerol and 0.5 % of oil was chosen as the best galactomannan coating.

6.4 CONCLUSION

The main objective of this chapter was to study the ability of chitosan and galactomannan to be used as coatings for cheese. Solutions of galactomannan (formulation: 1.5 % of galactomannan, 2.0 % of glycerol and 0.5 % of oil) and chitosan (formulation: 0.5 % of chitosan, 2.0 % of glycerol/sorbitol) were chosen based on their properties. It has been shown that a coating can be chosen based in its most relevant properties. Wettability has been considered the most relevant property mainly due to its ability to provide information about the behaviour of the coating on cheese surface.

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CHAPTER 7

EFFECT OF EDIBLE COATING APPLICATION ON GAS EXCHANGE AND SHELF-LIFE PARAMETERS OF CHEESE

The objectives of this chapter were determining the influence of the application of two different coatings (galactomannan and chitosan, developed in Chapter 6) and of storage temperature on the gas exchange rate of "Regional" cheese; subsequently, shelf-life parameters of coated and uncoated cheese were monitorized through the performance of chemical and microbiological analyses. Later, the selected coating was applied by different methods being the effectiveness of each application method evaluated.

7.1 INTRODUCTION

The use of coatings creates a modified atmosphere surrounding the commodity similar to that achieved by controlled or modified atmosphere storage conditions. The modified atmosphere created by edible coatings can protect the food from the moment it is applied, till its final retail destination and in the home of the consumer. As explained before many works have focused the use of polysaccharide-based coatings to extend and improve the shelf-life of fruits and vegetables, however only very few works used polysaccharide-based coatings to extend the shelf-life of cheese.

Cheese is the generic name for a group of fermented milk-based food products, being at the same time the most diverse group of dairy products. Cheese, in contrast with other dairy products, is biologically and biochemically dynamic and, consequently, inherently unstable (Fox & McSweeney, 2004). During the maturation and storage processes different reactions take place, influencing texture, flavour, and all the other chemical and physical properties of cheese (Pantaleão et al., 2007). Cheese protection through coating with synthetic films for moisture regulation and protection against contamination is a well-know procedure being cellophane, cellophane-polyethylene, saran, parakote, pliofilm, cryovac and aluminium foil some of the used coatings (Kampf & Nussinovitch, 2000). "Regional" cheese, used in the present work, is a semi-hard cheese sold with a polyvinyl acetate coating with the addition of a commercial antifungal agent.

In semi-hard cheeses, the factor that most affects cheese stability is water activity (a_w), which depends mainly on moisture and salt contents. During cheese ripening, a_w decreases until the surface is in equilibrium with the surrounding atmosphere, thus influencing the microbiological and chemical evolution of the cheese (Saurel et al., 2004; Robertson, 2006). Additional environmental factors must be considered when selecting a material for cheese coating (e.g. light, relative humidity, temperature, O_2 and CO_2 concentration). All these factors affect not only cheese's physical characteristics but also its flavour during storage. In fact, many different compounds contribute to cheese flavour and most of them are formed during cheese ripening (Robertson, 2006). During cheese storage biological reactions keep on occurring due to

microorganisms and enzyme activity, being the cheese quality influenced by the oxygen and temperature of storage, and gas exchanges with the environment. Cheese releases CO_2 and simultaneously consumes O_2 during its life cycle being required the control of the gas exchange to maintain the cheese quality and increase its shelf-life.

Application of edible coatings may be accomplished by dipping, spraying, brushing and individual wrapping (Donhowe & Fennema, 1994; Grant & Burns, 1994; Baldwin, 2007). When a product requires several applications of a coating to obtain uniformity on an irregular surface, or when cost is a factor, submerging the product into a tank of the emulsion may work best. After dipping and draining of excess coating, the film is allowed to set or solidify on the product (Donhowe & Fennema, 1994). To speed up the process, part of the process may entail the use of a drier to remove excess water or to dry the product under ambient conditions. When a thinner, more uniform film is required, spraying may be suitable for film application (Grant & Burns, 1994; Baldwin, 2007).

The present chapter evaluates, at different temperatures, the use of the previously optimized chitosan and galactomannan coatings (Chapter 6) to decrease O_2 consumption and CO_2 production rates of "Regional" cheese and to improve its shelf-life. The drying and application method of the coating was also studied.

7.2 MATERIALS AND METHODS

7.2.1 Raw material

Edible coating solutions were prepared with: chitosan (deacetylation degree of 90 % approximately, Aqua Premier Co., Thailand); galactomannan from *Gleditsia triacanthos* seeds (extracted as previously described); corn oil (Sovena, Portugal); glycerol 87 % (Panreac, Spain) and sorbitol 97 % (Acros Organics, Belgium); Tween 80 (Acros Organics, Belgium); lactic acid (Merck, Germany) and distilled water.

A commercial semi-hard cheese was obtained from Queijo Saloio S.A. (Portugal), being the samples stored at 4 °C until further use (approx. 5 days). "Regional" cheese is a full fat cheese produced with a mixture of caprine, bovine and ovine pasteurized milk; after being coated with synthetic coating (polyvinyl acetate) and antibiotic (natamycin), it is submitted to a short (aprox. 15 days) ripening period at low temperatures (5 °C and 12 °C in different stages of the ripening process). It requires storage conditions of 0-20 °C during retail. The cheese physicochemical composition is: moisture 46 % (w/w), fat 25 % (w/w), protein 18.4 % (w/w), total ash 3.58 % (w/w), chlorides 1.54 % (w/w), pH 4.8 and total acidity 1.40 ($g_{lactic\ acid}\ 100g_{cheese}^{-1}$) (Pantaleão et al., 2007).

7.2.2 Coating preparation

Previous work (Chapter 6) showed that polysaccharide edible coatings could be optimized having in consideration surface, permeability, colour and mechanical properties. This methodology has been applied in order to optimize coating solutions for "Regional" cheese; the optimum composition has been determined both for a solution of chitosan (0.5 % chitosan and 2.0 % glycerol/sorbitol) and galactomannan (1.5 % galactomannan, 2.0 % of glycerol and 0.5 % of corn oil (w/v)). The coating was prepared as described before (Chapter 6) and was stored at 4 °C until further use.

7.2.3 Coating application

The cheeses were coated with the solutions (of chitosan and galactomannan) by gently brushing their surface until all of it was covered, being the residual coating allowed to drip off. Then the cheeses were left during 4 h at 4 °C (92 % RH) until the coating was dry. For gas exchange measurements the whole cheese, with approximately 200 g, was used while for the monitorization of shelf-life parameters the cheeses were sliced in 30 g pieces, being the coating applied as described before. Different sample sizes were used due to the experimental setup

available for gas exchange measurements, where a cheese with approximately 200 g was needed to obtain the desired free container volume/total volume ratio.

7.2.4 O_2 and CO_2 exchange rates measurements

The closed system method was used for measurement of the gas exchange rate of the cheese. Several air-tight cylindrical containers of 0.14 m height were fabricated using 4 mm thick acrylic tube of 0.14 m diameter. The top and bottom of each container were covered with lids of the same material having the same thickness, being the top lid fitted with a septum for gas sampling. The acrylic container was considered impermeable to the gases (based on the supplyer's information). A whole intact cheese sample was placed in each container after equilibrating to the desired temperature. The change in gas composition (O_2 and CO_2 concentrations) inside each container was monitored with a gas analyser (Dansensor, Checkmate 9900, Denmark) during 7 days.

O_2 consumption and CO_2 production rates were determined applying Equations 7-1 and 7-2 (Salvador et al., 2002), developed for a closed system impermeable to gases.

$$R_{O_2} = -\left(\frac{dy_{O_2}}{dt}\right)\cdot\left(\frac{V_f}{w}\right) \qquad \text{Eq. 7-1}$$

$$R_{CO_2} = -\left(\frac{dy_{CO_2}}{dt}\right)\cdot\left(\frac{V_f}{w}\right) \qquad \text{Eq. 7-2}$$

where, R_{O2} is the O_2 consumption rate, mL[O_2] kg^{-1} h^{-1}, R_{CO2} is the CO_2 production rate, mL[CO_2] kg^{-1} h^{-1}, w (kg) is the weight of the cheese, V_f (mL) is the free volume of the container, calculated by:

$$V_f = V_p - \frac{w}{\rho_{ch}} \qquad \text{Eq. 7-3}$$

where, V_p (mL) is the total volume of the container, w (kg) is the weight of the cheese and ρ_{ch} is the true density of the cheese, in this case 1.095×10^{-3} kg mL^{-1} (Owolarafe et al., 2007). The graph of O_2 consumed *vs* time or CO_2 produced vs. time was used to calculate the slopes corresponding to the derivatives, dy_{O2}/dt (or dy_{CO2}/dt), that are directly proportional to the values of R_{O2} and R_{CO2} (please see Equation 7-1 and 7-2).

7.2.5 Temperature effects on the kinetics of O_2 consumption and CO_2 production

The Arrhenius equation (Eq. 7-4) was applied to study the effect of temperature on chemical reaction rates.

$$x = x_{ref} \cdot e^{\left(\frac{-E_{ax}}{R}\cdot\left(\frac{1}{T}-\frac{1}{T_{ref}}\right)\right)} \qquad \text{Eq. 7-4}$$

where x stands for a generic parameter, x_{ref} is the value of the generic parameter x at the arbitrarily chosen reference temperature, T_{ref} is the reference temperature (285 K), T is temperature, in K, E_{ax} is the activation energy for the parameter x, in kJ mol^{-1}, and R is the universal gas constant, 8.314×10^{-3} kJ mol^{-1} K^{-1}.

7.2.6 Cheese analysis

The weight loss was evaluated with a precision balance. The cheese was individually weighed at the beginning of the experiment and during the storage period, being the weight loss calculated by:

$$\Delta w = \frac{Iw - f\,iw}{Iw} \cdot 100 \qquad \text{Eq. 7-5}$$

where I_w is the initial weight and f_{iw} is the weight at the time *i*.

The moisture content was determined by measuring weight loss at 105 °C for 24 h (IDF, 1982).

Colour determination was performed using a Minolta colorimeter (CR 300; Minolta, Japan), where the changes in the surface colour of cheese samples were measured by the Hunter total colour difference (ΔE) expressed as L^* (whiteness or brightness/darkness), a^* (redness/greenness) and b^* (yellowness/blueness). The Hunter total colour difference (ΔE) was calculated by:

$$\Delta E = \sqrt{(L^* - L^*_0)^2 + (a^* - a^*_0)^2 + (b^* - b^*_0)^2} \qquad \text{Eq. 7-6}$$

where L^*_0, a^*_0 and b^*_0 are the initial values, obtained before packaging, and L^*, a^*, b^* are the values measured during the experiment.

The texture profile analysis (TPA) of cheese samples was performed with a TA.XT PlusTexture analyser (Stable Micro Systems, UK) with a load cell of 5 Kg and a 5 mm cylindrical plunger at a constant penetration speed of 2 mm min^{-1} (TPA) (three penetrations per cheese sample).

The pH value of each sample was recorded using a digital pH meter (3310, Jenway) equipped with a glass electrode that was inserted directly into the cheese sample for the measurement.

Microbiological analyses were performed by the determination of the total mesophilic count and total mould/yeast growth; in order to do this, cheese samples (20 g) were transferred aseptically to a stomacher bag, containing 180 mL of ringer solution (Merck, Germany) and the mixture was homogenized in a stomacher for 60 s. At each sampling day, two samples were analysed per treatment. Appropriate dilutions of the sample homogenates were prepared in sterile ringer solution and inoculated in duplicate in selective media: Plate Count Agar (PCA) (Merck, Germany) was used for the total mesophilic count and Potato Dextrose Agar (PDA) (Le Pont de Claix, France) was used to evaluate total mould/yeast count, being the spread plate technique used in both cases. PCA plates were incubated at 35 °C for 3 days and PDA plates were incubated at 25 °C for 5 days.

7.2.7 Design of experiments

The studied variables affecting gas exchange rate and chemical and microbiological analyses of cheese were temperature and the coating itself. Table 7-1 shows the settings (levels) used for each variable. For the gas exchange rate analyses a 3^2 design was applied, where the temperature (4, 12 and 20 °C) and the coating (without coating (NO), with chitosan (CH) and with galactomannan (GT) coating) were the independent variables. Three different temperatures within the storage temperature range provided by the manufacturer were used with the objective of evaluating the influence of the temperature in the cheese gas exchange rate analyses and in the shelf-life parameters.

For the cheese chemical and microbiological analyses a 2^2 design was applied, being the temperature (4 and 20 °C) and the coating (NO and GT coating) the independent variables. The relative humidity (HR) to the three used temperatures was 92 % HR to 4 °C, 78 % HR to 12 °C and 65 % to 20 °C. Experimentally determined quality parameters, during storage under different conditions, were analysed using Statistica 7.0 (Statsoft, Inc.). Pareto analysis was used to determine the environmental factors that were most significant in the changes of quality

parameters and sensory characteristics of cheese during storage. The Tukey test (α=0.05) was used to determine any significance of differences between specific means (SigmaStat, trial version, 2003, USA).

Table 7-1. Factors and levels used to measure RO_2, RCO_2 and shelf-life parameters of cheese

Experimental setup	Factors	Levels		
RO_2 and RCO_2	Coating	NO	CH	GT
	Temperature (°C)	4	12	20
Shelf-life parameters	Coating	NO	-	GT
	Temperature (°C)	4	-	20

7.2.8 Evaluation of drying and application method

The evaluation of the best temperature to dry the coating after being applied on the cheese surface was performed at three different temperatures (5, 20 and 35 °C). The temperature of 5 °C was chosen based on the temperature during the ripening period of the cheese (as used by the manufacturer). 20 °C was chosen as it corresponds roughly to room temperature, and 35 °C is the temperature used for film formation by casting. The relative humidity (RH) values for the three temperatures were 80 % RH at 5 °C, 50 % RH at 20 °C and 40 % RH at 35 °C. The dipping method was used to coat the cheese samples (based on preliminary tests that showed that this method is the one where a larger amount of coating stays on the cheese surface).

Cheese samples (30 g pieces) were weighted before coating, immediately after coating and after the drying operation, in order to estimate overall weight losses. The weight loss determination was performed as described in section 7.2.6. A linear relationship was obtained when plotting weight loss *versus* time, and the slope was used to obtain a weight loss rate (% h^{-1}). The drying temperature at which the highest differences between the slopes obtained for cheese with coating and cheese without coating were detected, was the one selected at this stage.

Three application methods were evaluated: dipping, brushing and spraying. The coating was considered dried after visual inspection and, as a limit, after the weight of the coated samples was equal to their initial weight before coating application. In the dipping method the samples were completely dipped into the coating solution for about 30 sec and then taken out, being the residual coating allowed to drip off. In brushing, the coating solution was gently brushed over each sample until all of it was covered, being the residual coating allowed to drip off. Spraying was performed using a paint sprayer (Proinsa, Spain) and the coating was applied at a 20 cm distance until the cheese was all covered, being the residual coating allowed to drip off.

The weight loss, moisture content and colour of the cheese were evaluated as described in section 7.2.6.

7.3 RESULTS AND DISCUSSION

7.3.1 O_2 and CO_2 exchange rates

To understand how coating and temperature influence the gas exchange rates, the cheese was coated using chitosan and galactomannan, and the values of RO_2 and RCO_2 were measured at 4, 12 and 20 °C. RO_2 ranged between 0.335 and 0.195 mL kg^{-1} h^{-1}; 0.540 and 0.375 mL kg^{-1} h^{-1}; and 1.45 and 0.635 mL kg^{-1} h^{-1} at 4, 12 and 20 °C, respectively, and RCO_2 varied between 0.265 and 0.125 mL kg^{-1} h^{-1}; 0.425 and 0.200 mL kg^{-1} h^{-1} and 2.250 and 0.900 mL kg^{-1} h^{-1} at 4, 12 and 20 °C respectively.

Figure 7-1 shows an increase of RO_2 with the increase of the temperature. The highest values were obtained for cheese samples without coating stored at 20 °C. The RO_2 values of uncoated cheese and of cheese with chitosan coating have a great increase (ca. 1.7-fold and 2.1-fold, respectively) when temperature is increased from 12 to 20 °C. However, from 12 to 20 °C, a smaller increase (ca. 0.2-fold) is observed in the RO_2 of the cheese coated with galactomannan coating, with a value ca. 80 % lower that the values of the other samples. Pareto analysis at 95 % significant level was used to quantify the effects of temperature and type of coating on RO_2

(Figure 7-2), showing that temperature, type of coating, interaction of both and the quadratic effect of the temperature affect significantly the values of RO_2.

Figure 7-3 shows that the increase of RCO_2 with the increase of the temperature is attenuated due to the presence of the coating. As for RO_2, it was also the galactomannan coating the one presenting the lower increase in CO_2 production when the temperature was increased from 12 °C to 20 °C. Pareto analysis (Figure 7-4) showed that temperature, quadratic effect of temperature, type of coating and interaction of temperature and type of coating affect significantly the values of RCO_2. As in the case of RO_2, also in RCO_2 the quadratic effect of the type of coating was negligible.

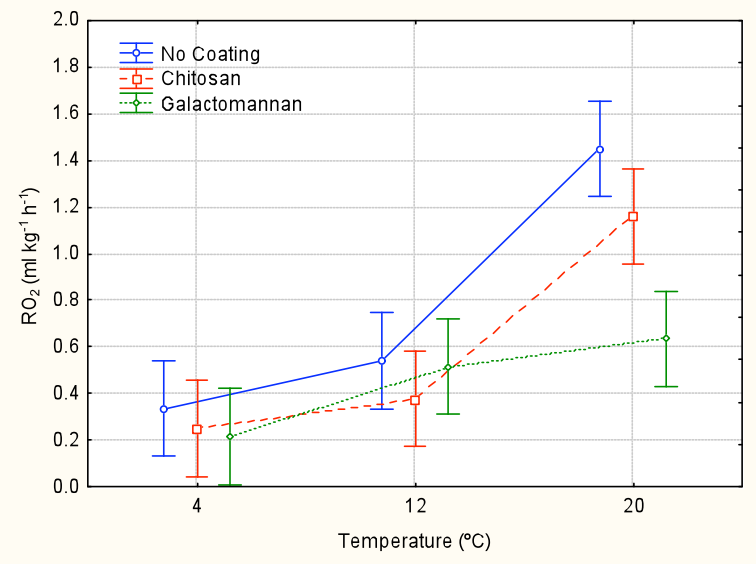


Figure 7-1. Interaction charts showing the effect of the temperature and coating in RO_2.

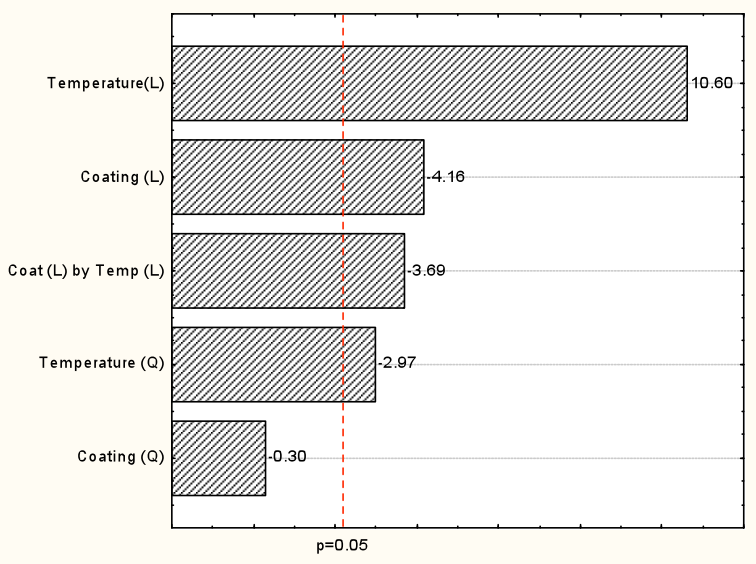


Figure 7-2. Pareto charts showing the effect of the temperature and coating in RO_2.

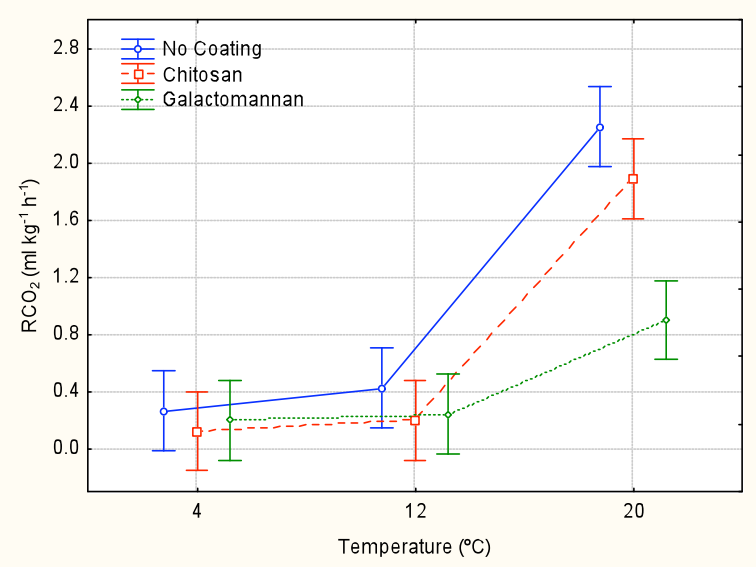


Figure 7-3. Interaction chart showing the effect of the temperature and coating in RCO_2.

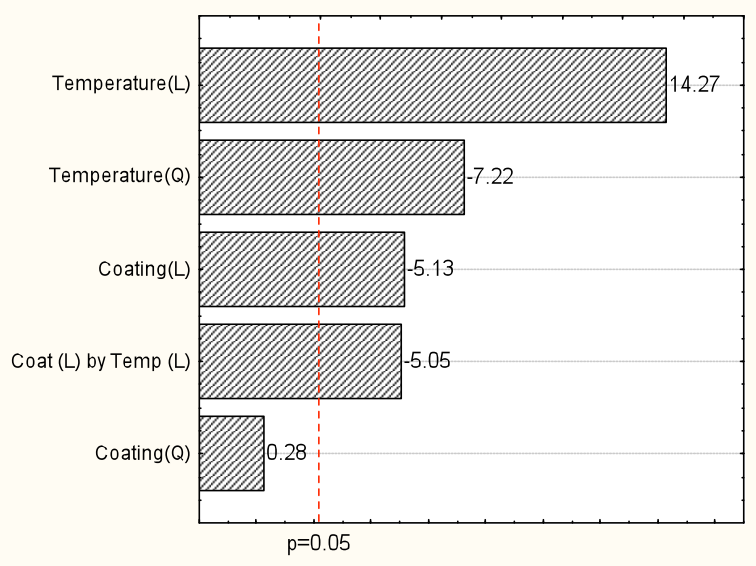


Figure 7-4. Pareto charts showing the effect of the temperature and coating in RCO_2.

O_2 consumption and CO_2 production are clearly temperature-dependent phenomena, and, with lower significance, type of coating-dependent phenomena. The very significant effect of temperature on cheese gas exchange was already observed by other authors (Fedio et al., 1994; Vivier et al., 1996), and has been attributed mainly to the growth of the microflora in cheese, that leads to increased values of O_2 consumption and CO_2 production (Robertson, 2006). The decrease of gas exchanges in the presence of coating are essentially due to the barrier properties of the polysaccharide coatings. The higher influence of the galactomannan coating in the values of RO_2 can be explained by its lower oxygen permeability (O_2P) (4.69×10^{-15} g Pa^{-1} s^{-1} m^{-1}), when compared with the value of O_2P for the chitosan coating (7.45×10^{-15} g Pa^{-1} s^{-1} m^{-1}) (Chapter 6). The values of RO_2 obtained in the present work were found to be very similar to those reported by other researchers, ranging between 1 and 2 mL kg^{-1} h^{-1} for Swiss cheese (Fedio et al., 1994) and Feta cheese (Vivier et al., 1996).

Different models describing the influence of temperature in the gas exchange rate have been reported in the literature, being the most common the Arrhenius equation which has been widely used to evaluate the effect of temperature on the gas exchange rate of different products (Fedio et al., 1994; Ratti et al., 1996; Andrich et al., 1998; Bhande et al., 2008). The Arrhenius model

has also been used to describe the dependence of temperature of other very complex reactions including bacterial growth and metabolism, and microbial death (Labuza et al., 1992). In this work the temperature influence in RO_2 and RCO_2 was well described by an Arrhenius equation (R^2>0.85), and the activation energy for gas production and consumption in "Regional" cheese was calculated from the slope of the linear plot log x *vs* $1/T$ (Eq. 7-4). Figure 7-5 shows the Arrhenius plots for some of the studied cases. The parameters of the obtained linear plot are shown in Table 7-2.

Cheese coated with the galactomannan solution has the lower values of activation energy, while cheese coated with the chitosan solution presents the higher values of activation energy. Despite the obtained lower values of RO_2 and RCO_2 for the cheese coated with chitosan coating at all the measured temperatures when compared with uncoated cheese, higher values of E_a are observed as consequence of a more significant effect of temperature on RO_2 and RCO_2. This increase can be explained by the lactic acid, used in chitosan coating formulation, that decreases the surface pH of the cheese thus favoring the increase of the yeast and moulds growth in cheese (McSweeney, 2007). All cheese samples tested have *Ea* values lower than the reported for Swiss cheese with values above 116.94 KJ mol^{-1} (Fedio et al., 1994).

Based on the RO_2 and RCO_2 exchange a coating was selected. The galactomannan coating was chosen in detriment of the chitosan coating due its better performance (lower gas transfer rate values) in terms of the gas exchange rates.

Table 7-2. Arrhenius equation parameters for RO_2 and RCO_2

	O_2 consumption			CO_2 production		
Cheese	Slope	R^2	E_a (kJ mol^{-1})	Slope	R^2	E_a (kJ mol^{-1})
NO	-7415.2	0.94	-61.6499	-10788	0.89	-89.6914
CH	-7863.1	0.92	-65.3738	-13898	0.85	-115.548
GT	-5618.7	0.89	-46.7139	-8423	0.87	-70.0288

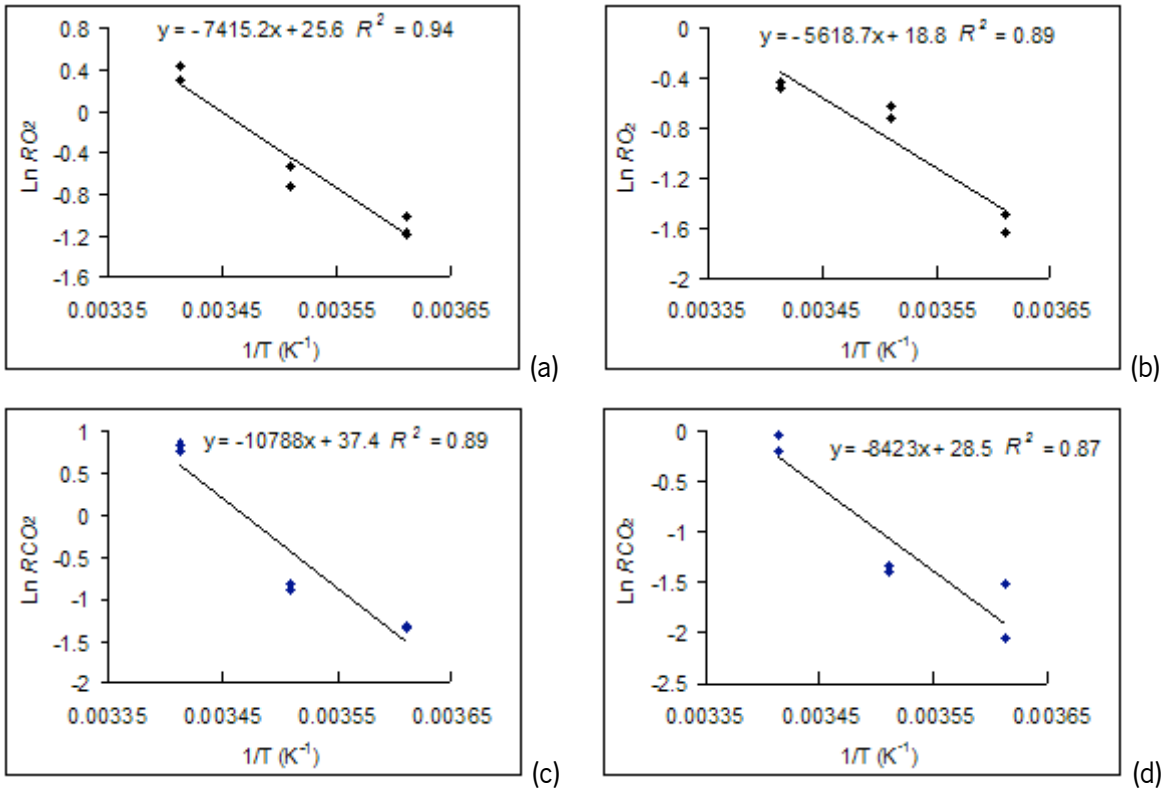


Figure 7-5. Arrhenius plots for RO_2 without coating (a) and with GT coating (b), and RCO_2 without coating (c) and with GT coatings (d).

7.3.1.1 O_2 and CO_2 exchange rates of unripened cheese after application of the galactomannan coating

"Regional" cheese after production and industrial coating process is submitted to a ripening period of approximately 15 days with two stages at low temperatures (5 and 12 °C). In order to evaluate the effect the coating in cheese before the ripening period (under extreme conditions, 22 °C), a galactomannan coating was applied in unripened cheese and gas transfer rates and weight loss were measured during 48 h.

The gas transfer rate results are presented in Figure 7-6. As observed before, the coated cheese clearly displays a lower gas exchange rate, being the CO_2 production rate higher than that of O_2 consumption. The obtained values of O_2 consumption rate ranged between 13.65 and

8.33 ml kg^{-1} h^{-1}, while the CO_2 production rate ranged between 14.52 and 9.27 ml kg^{-1} h^{-1} for uncoated and coated cheese, respectively.

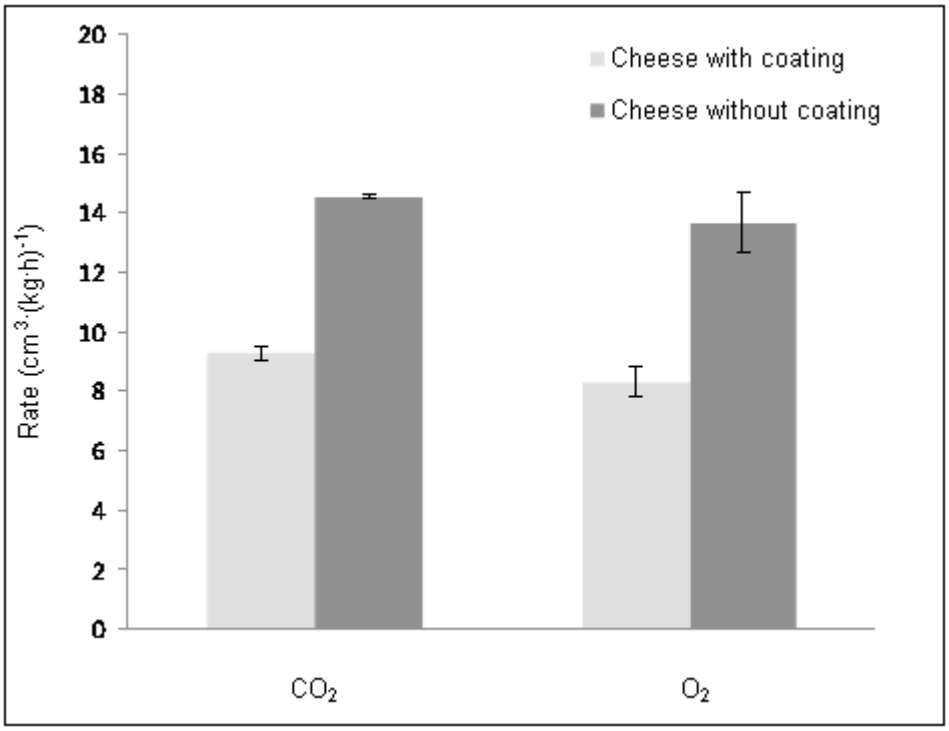
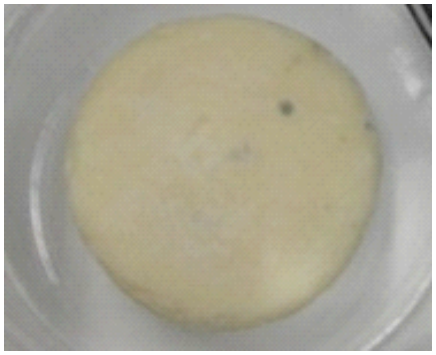


Figure 7-6. O_2 and CO_2 transfer rates in cheese at 21.86 ± 0.76 °C.

Previous work (section 7.3.1) shows values of RO_2 ranging between 1.45 and 0.635 mL kg h^{1} and of RCO_2 ranging between 2.250 and 0.900 mL kg^{-1} h^{-1}, at 20 °C, for ripened "Regional" cheese with galactomannan coating. As expected the physicochemical and microbial load of the cheese have a great contribution for the values obtained for unripened and ripened cheese. The presence of moulds in the surface of the cheese may also be related with the differences found: the coated cheese with less moulds at the surface (Figure 7-7) showed lower values of RO_2 and RCO_2. The coated cheese presents a relative weight loss of 0.11 ± 0.04 %, while the cheese without coating loses 0.84 ± 0.07 %. Therefore, the coating allows a decrease in the weight loss (ca. 8-fold the value in the absence of coating).

After 48 h from the beginning of the experiment, the cheese began to show fungal growth at the surface, mostly occurring on the uncoated cheese. Visual evaluation confirmed that the uncoated

cheese had extensive mould growth with almost the entire surface covered with mould colonies after only 48 h (Figure 7-7). The coating solution appears to have inhibited the growth of moulds, when compared with uncoated cheese.


(a)

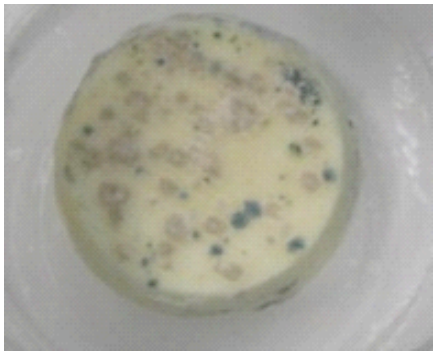

(b)

Figure 7-7. Cheese in jar, with coating (a) and without coating (b).

7.3.2 Chemical and microbiological analyses

In order to better understand the gas exchange rates phenomenon that happens in "Regional" cheese, chemical and microbiological analyses were performed comparing the uncoated cheese and the cheese coated with galactomannan coating. This coating was chosen in detriment of the chitosan coating due its better performance in terms of the gas exchange rates.

Weight loss analyses provided information on how the moisture loss during the storage period was influenced by the temperature and the presence of coating and if those factors can change the moisture loss profile of the cheese. Figure 7-8a shows the weight loss in the coated and uncoated cheeses for two temperatures (4 °C and 20 °C). In all cases the weight loss increased during the storage time, being the increases higher for the cheeses without coating (p<0.05). Pareto charts (Figure 7-9a) show that the presence of the coating is the only significant factor in the weight loss. Temperature does not seem to affect the moisture loss of the cheese during the 21 days of the experiment. These results showed that only the coating could retard water loss. These results are in agreement with those presented for alginate, gellan and k-carrageenan, used as coating materials on semi-hard cheeses. These were shown to present a lower weight loss

(approximately 2.0 %) when compared with uncoated cheese samples (Kampf et al., 2000). The values for moisture loss after 21 days ranged between 23.4 % and 19.6 % for uncoated and coated cheeses, respectively. These values are in agreement with weight loss results, and Figure 7-8b shows that the moisture content decreases in all cases, being higher for the samples without coating stored at 20 °C. At the end of 21 days of storage the cheese samples have moisture content values between 15.3 % and 13.0 % for coated cheeses at 4 °C and 20 °C respectively, and 12.8 % and 11.1 % for uncoated cheeses samples at 4 °C and 20 °C, respectively.

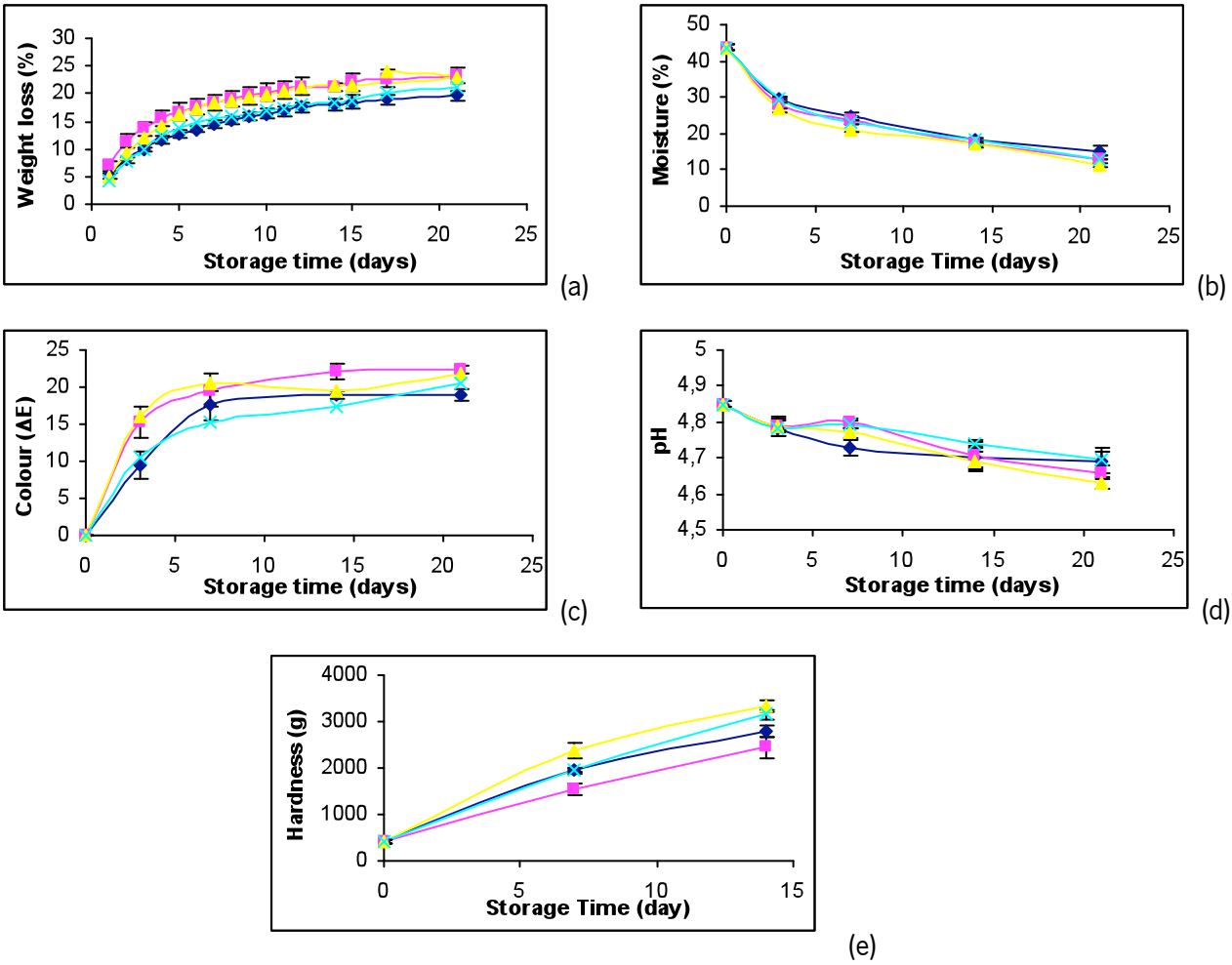


Figure 7-8. Average and standard deviation throughout storage time for cheese without coating ■ and with coating ◆ at 4 °C, and without coating ▲ and with coating × at 20 °C in terms of: weight loss (a), moisture (b), colour difference (ΔE) (c), pH (d) and hardness (e).

Pareto charts (Figure 7-9b) show that temperature and the presence of coating are factors affecting significantly the moisture content. Pantaleão et al. (2007) showed that the decrease of the water content of "Regional" cheese using *Humidipak* and microperforated packaging systems can be less pronounced, with higher values of moisture content after 21 days of storage. These differences can be explained by the weight of the cheese sample used in our work, lower than the one used in Pantaleão et al. (2007). Both weight loss and moisture loss are related to the water loss of the cheese that depends on the kinetics of water permeation through the coating used (Robertson, 2006). The presence of the coating decreased the moisture loss of the cheese in 2.5 % and the weight loss in 3.8 % at 4 °C, while at 20 °C the moisture loss and the weight loss decreased 1.9 % and 3.1 %, respectively.

Colour analysis, based in the colour difference (ΔE), showed that the uncoated cheese at 20 °C has higher values of ΔE. Figure 7-8c shows that the differences between uncoated and coated cheese during the storage time become more and more significant ($p<0.05$) during the first 3 days. The Pareto chart (Figure 7-9c) shows that the presence of coating, the temperature and the interaction between the presence of coating and the temperature are significant factors affecting the colour difference. The cheese storage using a coating and the decrease of the temperature can be used to decrease colour changes, with clear advantages in terms of the marketing of the cheese. The influence of temperature in colour change was also reported for Mozzarela cheese where the whiteness parameter (*L*-value) increased with the cheese heating (Metzger et al., 2000). Colour change can be essentially attributed to cheese oxidation, that is lower in cheeses with coating due to the protection from the oxygen and light oxidation provided by the coating's O_2P and opacity.

After 14 days of storage, the pH became regular in cheese samples, without statistically significance between samples ($p>0.05$) (Figure 7-6d). pH presented values ranging between 4.85 and 4.63 and Pareto charts showed that no factor was significantly affecting pH variation after 21 days (results not shown). Figure 7-8e presents the hardness values for cheese samples during the storage time, showing that coated cheeses stored at 4 °C have the lower hardness values. The Pareto chart (Figure 7-9d) shows that cheese hardness (checked in days 0, 7 and 14), has the temperature as the most important factor, being the interactions between the temperature and the presence of a coating also significant effects (Figure 7-9d). The hardness values

measured in the present work were higher than those reported in Pantaleão et al. (2007); this is possibly due to the higher water loss measured in our cheese samples and also to the different cylindrical plunger used in this work, which makes the comparison somewhat tricky.

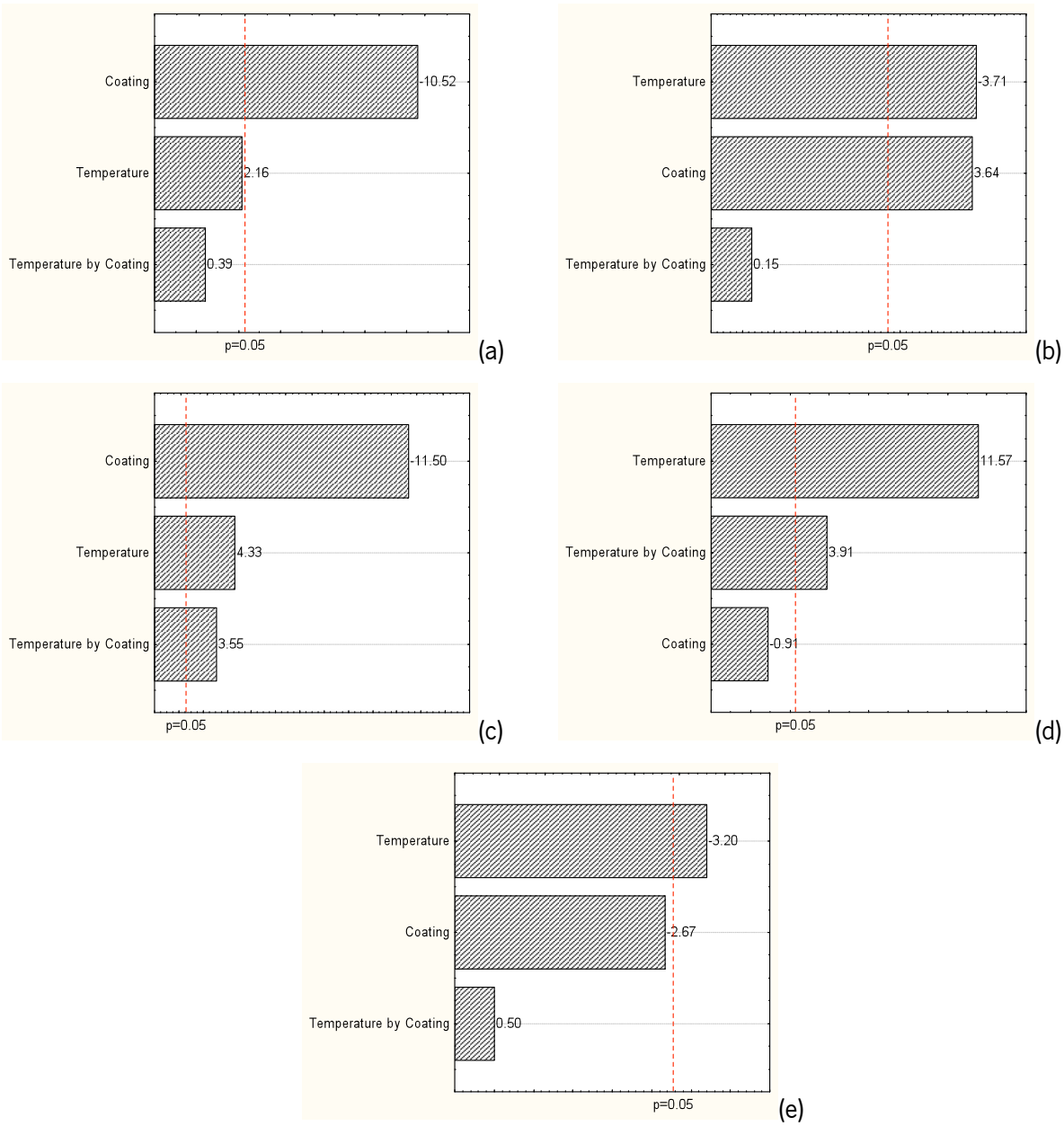


Figure 7-9. Pareto charts of the effects obtained from the fractional factorial design in the last (21st) day of analysis for: weight loss (a), moisture (b), colour difference (ΔE) (c), hardness (d) and total mesophilic count (e).

Microbiological analyses of cheese samples (Table 7-3 and 7-4) presented a smaller increase of the CFU g^{-1} in coated cheese. In Table 7-3, the total mesophilic bacteria count showed a great increase for all samples in day 7, being a decrease observed between the days 14 and 21. Similar results were observed for the total molds/yeast counts. These results are in line with the values of RO_2 and RCO_2 of the cheese (measured during 7 days), where at 4 °C no statistical difference (p>0.05) is found between RO_2 and RCO_2 of the uncoated and galactomannan coated cheese, this not being the case at 20 °C temperature at which a statistical difference (p<0.05) was observed (see Figures 7-1 and 7-3).

Table 7-3. Total microbial count log(CFU g^{-1}) of cheese samples as a function of the day of storage

Coating	Temperature	Day			
		0	7	14	21
NO	4 °C	5.3±0.2^{a}	6.7±0.4^{ab}	5.9±0.5^{a}	6.5±0.3^{a}
	20 °C	5.3±0.2^{a}	7.0±0.1^{a}	6.7±0.2^{b}	5.9±0.1^{bc}
GT	4 °C	5.3±0.2^{a}	6.6±0.3^{ab}	5.2±0.4^{a}	6.1±0.1^{ab}
	20 °C	5.3±0.2^{a}	6.4±0.2^{b}	5.7±0.2^{a}	5.7±0.2^{c}

$^{a-c}$Different superscript letters in the same column indicate a statistically significant difference (Tukey test p<0.05).

Table 7-4. Total moulds/yeast count log(CFU g^{-1}) of cheese samples as a function of the day of storage

Coating	Temperature	Day			
		0	7	14	21
NO	4 °C	5.6±0.0^{a}	6.6±0.0^{a}	5.9±0.2^{a}	5.9±0.2^{ab}
	20 °C	5.6±0.0^{a}	6.9±0.1^{b}	6.0±0.3^{a}	5.8±0.0^{a}
GT	4 °C	5.6±0.0^{a}	6.6±0.0^{a}	5.6±0.2^{a}	6.1±0.1^{b}
	20 °C	5.6±0.0^{a}	6.0±0.2^{c}	-	5.4±0.2^{c}

$^{a-c}$Different superscript letters in the same column indicate a statistically significant difference (Tukey test p<0.05).

The presence of coating decreases significantly (p<0.05) the growth of mesophilic aerobic bacteria in days 7 and 14 for cheese samples stored at 20 °C. A similar result is observable when counting the total moulds/yeasts (Table 7-4) in day 7 and day 21 at 20 °C. The Pareto chart (relative to the values obtained in the day 21 – Figure 7-9e) showed that total mesophilic bacteria numbers are influenced by the temperature, only. The smaller increase in the microbial counts on cheese coated with galactomannan coating can be explained by the reduced gas permeability, as confirmed by the values RO_2 and RCO_2, of this coating that decreases the oxygen transfer rate into the cheese, that becomes less available for the growth of mesophilic bacteria, moulds and yeast. The counts obtained for mesophilic and mould/yeast ranged between 5.3-7.0 and 5.6-6.9 log(CFU g^{-1}), respectively, and are in agreement with other works with mozzarella, Turkish white and Cheddar cheeses (Öner et al., 2006; Duan et al., 2007; Suppakul et al., 2008).

7.3.3 Effect of the drying and application method in the efficiency of the coating

7.3.3.1 Effect of the drying temperature

When coatings/films are applied directly on foods, they need to dry so that the formation of the film takes place successfully. The drying of the coating is influenced by temperature and relative humidity. In general, reported conditions of drying range from a few minutes to 12 h at temperatures of 20, 30 and 40 °C (Kampf et al., 2000; Higgins, 2003; Smits & De Haan, 2006; Duan et al., 2007).

In this work three temperatures were tested (5, 20 and 35 °C) in order to evaluate the time necessary for the coating to dry. To understand how the temperatures can influence the cheese weight loss during the drying process, cheese without coating was submitted to the same drying temperatures of the coated cheeses. The best drying temperature was chosen based on the weight loss of the coated cheese as compared to that of cheese without coating. This approach was used in order to relate two important mechanisms during the drying process: drying of the coating itself and the weight loss of the cheese.

Table 7-5 shows the time necessary for drying the coating at each temperature used. The drying period was of 60, 90 and 240 min, for the temperatures of 35, 20 and 5 °C, respectively. As expected, the increase of the drying temperature leads to higher values of cheese weight loss, and consequently the slope obtained when plotting weight loss *versus* time increases. It can be observed that cheese with coating presents a higher weight loss rate, which has been related with the evaporation of the water from the coating solution on the cheese surface (Table. 7-5).

The slopes presented in Table 7-5 provide an indication of the weight loss rate and can be used to predict the drying temperature that simultaneously allows the drying of the coating and minimizes the weight loss of the cheese. This is obtained by the ratio between the slope of the curve "weight loss of the cheese with coating" *versus* "time" (Slope GT coating) and the slope of "weight loss of the cheese without coating" *versus* "time" (Slope NO coating), for each temperature. The highest value was obtained at 20 °C, followed by 5 °C and 35 °C. The temperature of 35 °C leads to a higher weight loss of the cheese without coating.

Table 7-5. Drying time for the coatings at each temperature used; slopes of the corresponding weight losses of the coating itself and of the cheese without coating

Temperature (°C)	Time (min)	Slope GT coating (% h^{-1})	Slope NO coating (% h^{-1})	Slope GT/slope NO
5	240	0.01405	0.00989	1.42
20	75	0.06012	0.03894	1.54
35	60	0.07678	0.05996	1.28

7.3.3.2 Effect of the application method

In order to understand how the application method can influence the coating performance, three methods were tested: dipping, brushing and spraying. During coating application the effect of the application method on the cheese weight gain and in the coating used on cheese covering was

studied. Properties as the weight loss, moisture content and colour changes were also performed.

During coating application the excess of coating is allowed to drip off, with the consequent loss of coating material; depending of the method used, this loss can differ. Table 7-6 shows the amount of coating used per cheese sample for different application methods. Is evident that the amount of coating depends on the application method, being brushing the one where a lower amount of coating is spent and, consequently, where the weight gain of the cheese after the coating application is the lowest. Spraying is the method with the greatest consumption of coating solution; however, it does not present the highest cheese weight gain.

The most efficient method was the dipping method, where the efficiency in terms of coating retainment on the cheese surface was the highest of all tested methods.

The thickness of the coating after the drying process was evaluated for all the application methods, and the cheese surface was observed using a magnifying lens (Olympus, USA). It was observed that spraying and dipping methods present the most regular surface coating; in all cases the coatings present thickness values lower than 0.01 mm.

Table 7-6. Spent and wasted coating (through drip losses), cheese weight gain and calculated efficiency of application for each method of coating application

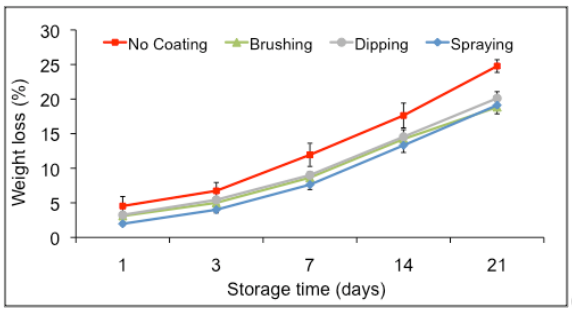
Application method	Spent coating (g cheese^{-1})	Cheese weight gain (g cheese^{-1})	Wasted coating (g cheese^{-1})	Efficiency (%)
Dipping	4.98±0.64	1.71±0.35	3.27	34
Brushing	2.40±0.87	0.45±0.13	1.94	19
Spraying	8.21±0.35	0.92±0.13	7.72	11

In order to understand how the application method can influence the shelf-life parameters of cheese during storage, the weight loss, moisture content and the colour parameters were evaluated during 21 days. Figure 7-10a shows the weight loss of coated and uncoated cheeses. In all cases it increased during the storage time, being the increases higher for the cheeses without coating (p<0.05). The presence of the coating decrease the weight loss, as already observed before; however, in this case the differences between the uncoated and coated cheeses increased. After 21 days of storage the uncoated cheese presents a weight loss of 24.8 % while for coated cheese the weight loss presents values of 20.1, 19.1 and 18.8 % for dipping, spraying and brushing, respectively. These values are in agreement with the values obtained for moisture (Figure 7-10b), where the uncoated cheese presents values of 25.6 % and the coated cheese displays values always above 29.1 %. No statistically significant differences (p<0.05) were detected between cheese samples with different application methods, however brushing and spraying present the lowest values for weight loss and moisture, respectively.

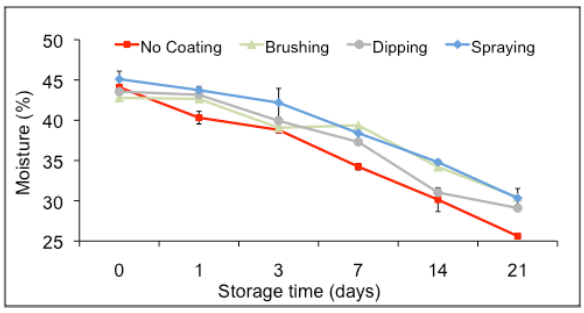
Colour analysis, based in the colour difference (ΔE), showed that uncoated cheese presents the highest values of ΔE (Figure 7-10c) after 21 days of storage. The spraying method shows to be the most efficient to decrease the colour differences during storage, however this happens without a statistically significantly difference (p<0.05) from the other coating application methods after the 21 days.

Results show that the application method apparently does not have a statistically significant influence in the efficiency of the coating in terms of cheese weight loss, moisture loss and colour differences. However, having in consideration the spent and/or wasted coating during coating application by the different methods, brushing or dipping would be the ones to choose.

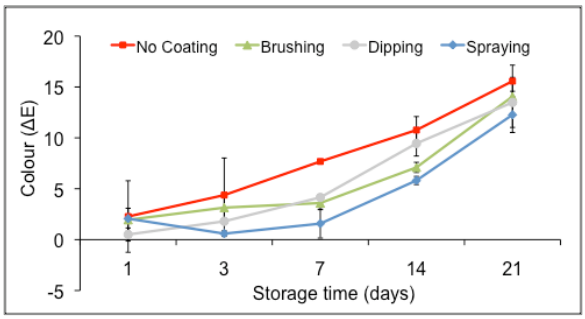
It was also observed that spraying presents, for all the evaluated parameters, the best results, with lower values of weight loss and moisture content, and the lowest colour difference on the cheese.



(a)



(b)



(c)

Figure 7-10. Average and standard deviation throughout storage time for cheese without coating and with coating (applied by brushing, dipping and spraying) in terms of: weight loss (a), moisture (b) and colour difference (ΔE) (c).

7.4 CONCLUSION

Cheese samples without coating presented the highest values of RO_2 and RCO_2 for all temperatures analyzed. Temperature had an important effect on RO_2 and RCO_2, and the cheese with galactomannan coating presented the lowest value of *Ea*, thus showing the positive influence of the galactomannan coating in the decrease of the respiration rates in cheese. Based in the obtained results for RO_2 and RCO_2, the galactomannan coating providing the lowest gas exchange rates was applied on cheese samples and chemical and microbiological analyses were performed. It may be concluded that the application of a galactomannan coating together with 4 °C temperature can be used to improve "Regional" cheese's physicochemical parameters after 21 days of storage. Dipping and brushing seem to be the most adequate options for coating application on cheese samples if coating delivery efficiency is the parameter to maximise.

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CHAPTER 8

GENERAL CONCLUSIONS

This chapter presents the major conclusions of this thesis and advances recommendations/suggestions for further research in this field.

8.1 CONCLUSIONS

The main objective of this thesis was the development and characterization of new edible coatings for the preservation of cheese quality. In order to cover successfully the thesis aims, several subjects were studied and strategies were implemented. In particular, the work involved the characterization of galactomannans from new sources; the study of the utilization of polysaccharides sources as edible coatings/films and how they where influenced by the addition of plasticizers and oil; and the selection of a coating/film formulation based on their wettability, and physical properties. The main contributions of this thesis were the following:

- A methodology for the extraction and purification of galactomannans from seeds of three different species, which allowed to obtain galactomannans from non-traditional sources. This method also shows to be simpler and easier to perform than most of the published procedures, while also avoiding the use of non-food grade solvents;
- Physicochemical characterization of galactomannans confirms the suitability of these sources to be used in food industry;
- Galactomannans properties showed to be highly influenced by mannose/galactose content; *Gleditsia triacanthos* presented a different pattern with the presence of acetyl and pentose groups in the mannan backbone;
- During galactomannans extraction procedures it is possible to obtain phenolic and antioxidant compounds from the seeds. These phenolic and antioxidant extracts have shown ability to be incorporated in galactomannan films, confering functional properties to the films;
- The presence of glycerol and corn oil in chitosan and galactomannan coatings/films showed to influence their properties. The effects of glycerol and corn oil on properties such as: wettability, moisture content, solubility, water vapour permeability, mechanical properties and opacity were shown, being the main differences supported by the water affinity of the components of the coatings/films constituents, being confirmed by Fourier transform infrared spectroscopy, differential scanning calorimetry and thermogravimetric analyses;

- Chitosan and galactomannan coatings/films showed to have different physicochemical properties related with the polysaccharides structures, being the main factors the charged and neutral structure, and the presence of the NH groups in chitosan;
- Edible films present lower values of tensile strenght than common plastic films, while their elongation-at-break varies widely, presenting in some cases elongation-at-break values comparable to those of common plastic films;
- The wettability of the coatings on the cheese surface was selected as the most important property to be evaluated when an efficient cheese covering is the main objective;
- The application of chitosan and galactomannan coatings on cheese samples and their storage temperature showed to influence the gas exchange rate of "Regional" cheese, being the galactomannan coatings the ones that allow a lower gas transfer rate;
- Galactomannan coatings and the storage temperature at 4 °C have shown to be a good option in order to improve shelf-life parameters of "Regional" cheese, being a good alternative in order to replace the synthetic coatings;
- Different drying and application methods showed that, despite presenting similar performances, when referring to cheese moisture loss they can present different application efficiencies, being brushing the application method where less coating is wasted;
- A methodology where the coatings/films were evaluated in order to choose the formulation that leads to the better performance to coat the cheese, which can be applied to other food products, as e.g fruits and vegetables.

Briefly, galactomannans from non-traditional sources present good properties to be used as edible coatings/films, and for the incorporation of functional compounds. A new methodology showed how a coating can be selected by its properties, being a guide for the use of new coatings for cheese as alternatives to synthetic coatings, or on cheese where the coating do not exist. This work may also be a guide for the study of future new materials for similar purposes.

8.2 RECOMMENDATIONS

Despite the main objectives have been achieved, some work still stays to be done in order to better understand the properties of polysaccharide edible coatings/films, and how can they be used to replace commercial synthetic plastics. Thus, some recommendations for improvement of the present work and guidelines for future work in this field can be advanced:

- Upon extraction of the functional compounds from *G. triacanthos* seeds, their phenolic content and antioxidant capacity have been shown, however more studies should be performed in order to indentify the main compounds responsible for such activities;
- Research on the utilization of the methods presently used for synthetic packaging film formation (e.g. injection moulding, film blowing or extrusion) to obtain galactomanann and chitosan films, in order to evaluate how this methods can be used for production of edible films;
- Study of the cheese shelf-life parameters using simultaneously the application of coatings and films as wrapping, providing a more effective/realistic modified atmosphere packaging;
- Finally, the evaluation of the selected coating formulation in an industrial environment, in order to understand the behaviour of these edible coatings on cheese during an industrial scale application.